Analysis of physiological partners of protein kinase CK2 in Drosophila melanogaster

Umesh C. Karandikar

West Virginia University

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Analysis of physiological partners of protein kinase CK2 in *Drosophila melanogaster.*

**Umesh C. Karandikar**

Dissertation submitted to the Eberly College of Arts and Sciences at West Virginia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

Ashok Bidwai, Ph.D., Chair
Philip Keeting, Ph.D.,
Clifton Bishop, Ph.D.,
Kristine Krajnak, Ph.D.,
Stephen Graber, Ph.D.,

Department of Biology
Morgantown, West Virginia
2005

**Keywords:** CK2, SSL, E(spl) M8, Atonal, Notch

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Abstract

Analysis of physiological partners of protein kinase CK2 in

*Drosophila melanogaster.*

Umesh C. Karandikar

CK2 is a highly conserved Ser/Thr protein kinase composed of catalytic (α) and regulatory (β) subunits. The enzyme targets proteins that are involved in a variety of cellular processes such as DNA replication, transcription, translation, cell cycle progression, and development. However, very little information is available on either the regulation of this enzyme or its role during development. These two aspects of CK2 have been addressed using the fruit fly, *Drosophila melanogaster,* as a model organism. The first study (chapter 2) addresses the isolation and characterization of SSL, an ortholog of CK2β. Using combinatorial approaches, we find that SSL associates with CK2α via a domain that is indistinguishable from that in CK2β. In addition, we find that SSL functionally mimics the biological properties of CK2β, but exhibits an expression pattern that is non-overlapping, raising the possibility that SSL might alter the specificity of this enzyme. The second study (chapters 3 and 4) addresses the developmental role of CK2 with respect to the phosphorylation of the neural repressor E(spl)M8. We find that phosphorylation of M8 (by CK2) is essential for its ability to mediate transcriptional repression during eye development. This repression is essential for the precise positioning of neurons in the developing Drosophila retina. Using a combination of transgenics, cell-fate mapping, and mutant analysis, we implicate a role for this enzyme and identify a critical molecular target (Atonal) of phosphorylated M8. The mechanism we describe is now also implicated in the mammalian retina, where CK2 mediates the phosphorylation of Hes6, the M8 homolog. We have extended our understanding of the roles of this enzyme during neurogenesis by biochemically characterizing a novel target, Deadpan (chapter 5). Taken together, these studies have uncovered a novel evolutionarily conserved developmental role for CK2 as a regulator of neurogenesis.
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Chapter 1.

Introduction
1. A brief history of Protein kinases

During the development of metazoans a single cell (a fertilized egg) develops into a multicellular organism with a bewildering array of organs, each containing a precise spatial arrangement of diverse cell types. This systematic transformation in complexity is achieved through an intricate interplay of communications that have been collectively termed as ‘signal transduction’. Analysis of signal transduction in taxa representing diverse phylogenetic groups such as yeast, insects, amphibians, and mammals have demonstrated an astonishing level of conservation throughout evolution. While single cell eukaryotes display a much simpler repertoire of signaling pathways, their number and complexity increases dramatically in metazoans. In addition, development of a metazoan organism often employs the same signal transduction pathways in multiple contexts.

Cell-cell communications are mediated through multiple mechanisms during development. These include soluble factors such as hormones that can either act at long distances (endocrine) or act in a localized/restricted fashion (paracrine/autocrine). Irrespective of the nature of the signal or its source, these communications invariably involve cellular mechanisms such as, the coordinated release of specific (secondary) signaling molecules (the second messengers), the targeted activation/inactivation of specific proteins, or the targeted expression/repression of genes. The mediators of these processes prominently include members of highly conserved protein families. Notable among these are protein kinases (receptor and non-receptor), protein phosphatases, phospholipases, G-proteins, adaptor proteins, etc. I restrict my discussion to the roles of protein kinases during the process of cell signaling.

Protein kinases represent, perhaps, the largest family of evolutionarily related, but functionally distinct, regulatory proteins that have been identified in organisms as diverse as prokaryotes and eukaryotes. For the sake of brevity and simplicity, I have focused on the eukaryotic members of this superfamily. The basic reaction catalyzed by protein kinases is mechanistically similar, i.e., the transfer of the $\gamma$-phosphate group of either ATP or GTP to the hydroxyl group of an amino acid such as serine, threonine or tyrosine of a protein substrate. In this case, the specificity of phosphorylation is achieved by the selective activation of a protein kinase, and the targeting of proteins via the recognition of unique and
non-overlapping consensus sequences. As a result, activation of a particular protein kinase elicits unique changes in the cellular phosphoproteome.

The seminal studies of Edwin Krebs and Edmond Fischer in 1955 demonstrated, for the first time, how phosphorylation serves to regulate protein (enzyme) activity (Fischer and Krebs, 1955). During their efforts to purify glycogen phosphorylase (a key enzyme required for hydrolysis of glycogen) they observed inconsistent activity of the purified enzyme. In a moment of serendipity, they had stumbled upon an enzyme that could be converted from an active to an inactive form. During studies to understand the basis of this ‘activity conversion’, they uncovered that phosphorylation of this enzyme was necessary for its activation. The enzyme responsible for this phosphorylation was itself activated by a second messenger, cAMP. This enzyme is now known as the cAMP-dependent protein kinase (PKA). Since their landmark discovery, an astonishing number of enzymes catalyzing similar reactions have been discovered and characterized in all eukaryotes. It has since been found that protein kinases regulate virtually all aspects of cellular biology such as cell division, DNA replication, gene expression, cell polarity, and extends throughout the process of animal development from the time point of embryogenesis until senescence. The profound implications of their findings and their widespread importance to biology, in general, led to their recognition with the award of the Nobel prize in Medicine and Physiology in 1992.

Over the last 50 years, a variety of protein kinases have been purified and characterized. Some of these specifically target Ser/Thr residues and are called the Ser/Thr Protein Kinases. In contrast, another superfamily has been discovered that selectively targets Tyr residues, and members of this group are thus called the Tyrosine Kinases. A third class of these enzymes has been identified and these target Ser/Thr and Tyr, reasons for which these members are called the ‘switch hitters’. The importance of Tyrosine Kinases to the process of cellular transformation was also serendipitously discovered by Michael Bishop and Harold Varmus, who found that the viral gene responsible for formation of sarcomas, v-Src, encoded a protein kinase which also existed in its host cell as a cellular version called c-Src. They found that the c-Src Protein Kinase was under strict cellular controls, whereas that encoded by the viral genome (v-Src) escaped proper cellular regulation and elicited oncogenic transformation (Varmus et al., 1989). These studies established the potential of
protein kinases to serve as ‘proto-oncogenes’. The profound implications of their findings were recognized by the award of the Nobel Prize in 1989. It was, therefore, not entirely surprising when it was discovered that the process of cell division was, itself, subject to tremendous control of the activities of a variety of such enzymes. The elegant analysis in yeast and in sea urchins, by Leland Hartwell, Paul Nurse, and Tim Hunt uncovered just such a control mechanism. Their identification of a *cdc* (cell division cycle) mutant in the budding and fission yeast called *cdc28* a.k.a. *cdc2*, was linked to process of cell division (mitosis) with the independent discovery by Tim Hunt that *cdc2* is the protein kinase component of the ‘maturation promoting factor’, now well known as a Cyclin-dependent Kinase (CDK) (Hunt, 1989; Nurse et al., 1998). The elaboration of the biochemical circuits that orchestrate this fundamental aspect of cell biology and its implications to the process of oncogenesis, led to all three sharing the Nobel Prize in 2001.

2. Protein kinase CK2:

In 1954, the laboratory of Eugene Kennedy described the existence and partial purification of an enzyme (“protein phosphokinase”) that catalyzed the phosphorylation of a model substrate, casein, using ATP as phosphoryl donor (Burnett and Kennedy, 1954). The choice of casein as an inexpensive, readily available protein, in all likelihood, sealed his fate in scientific history. Unable to uncover any biological effects of this phosphorylation, he decided to abandon this area of research, and instead pursue the area of lipid biosynthesis. It was not until the landmark paper by Krebs and Fischer was published in 1956, that Eugene Kennedy recognized what he had, in fact, discovered. In a ‘perspectives’ commentary he stated, “Like the base Indian—I cast away a pearl far richer than all my tribe” (Kennedy, 1992). The enzyme that Eugene Kennedy described is thus the first protein kinase ever identified, and a close examination of the methods he described in this manuscript indicates that he had, perhaps, discovered the ‘casein kinases’ (Burnett and Kennedy, 1954). Unfortunately, because phosphorylation of casein was not the biological function of this group of intracellular enzymes and owing to the unfortunate nomenclature, this group of enzymes was to a large extent ignored. It was not until 1979, that the laboratory of Jolinda Traugh succeeded in the isolation and purification two distinct protein kinases from calf
adrenal cortex, which she called Casein Kinase I and Casein Kinase II (Hathaway and Traugh, 1979). Since then, these two enzymes have been demonstrated to be highly conserved and extensive biochemical, molecular, and genetic analysis of their functions have revealed that they are distinct entities with distinct biological functions (see below). Given their importance to cell/organismal biology and to avoid confusion with regard to the term ‘casein’, a decision was made to rename this group of enzymes as CK1 and CK2.

2.1. Biochemical Properties of CK2:

CK2 has been purified to homogeneity from a variety of organisms, and in each case it has been found to exhibit a tetrameric conformation (the holoenzyme). The holoenzyme is comprised of two catalytic subunits (CK2α) and two regulatory subunits (CK2β). This composition is seen for this enzyme from unicellular eukaryotes (yeast) and all metazoans (insects, amphibians, and mammals) (Allende and Allende, 1995; Pinna, 1994; Pinna, 2002).

From the time of its discovery, a substantial amount of effort has been devoted to describing the biochemical and biophysical properties of CK2. These include an ability to phosphorylate Ser/Thr, an ability to utilize either ATP or GTP at almost equivalent efficiencies (Rodnight and Lavin, 1964), an activity that is modulated by ionic strengths, inhibition by heparin (Dahmus et al., 1984; Glover et al., 1983; Hathaway et al., 1980; Meggio et al., 1982), and activation by polybasic compounds such as spermine, protamine and polylysine (Bidwai et al., 1993; Meggio et al., 1987). The ability to utilize ATP or GTP is, generally, uncommon in most kinases, as is the inhibition by heparin. In addition, incubation of purified CK2 with [γ³²P]ATP results in the incorporation of ³²P into CK2β, a reaction termed ‘autophosphorylation’ (Meggio et al., 1983; Meggio and Pinna, 1984). This property is independent of the source of CK2. This autophosphorylation reaction is inhibited upon activation of the enzyme by the polybasic activator poly(DL)Lysine. At face value, this suggests that autophosphorylation is antagonistic to catalysis. In this regard a comparison to the Calmodulin-dependent Protein Kinase (CamKinase II) would be illustrative. In the case of this enzyme, autophosphorylation obviates its dependency on Ca²⁺ and Calmodulin, alters subcellular localization, susceptibility to proteolysis, etc. (Hudmon and Schulman, 2002). This, however, is not the case for CK2 because biochemically the autophosphorylated enzyme displays proper phosphorylation of its physiological targets. This by no means is
evidence arguing against any role for autophosphorylation, because non-phosphorylatable alleles of CK2β are compromised for function in vivo (see below). As mentioned above, CK2 is a α2β2 tetramer, a conformation highly reminiscent of PKA (reviewed in Taylor, 1989), which also exists as an R2C2 tetramer (Fig. 1). In the case of the PKA tetramer, cAMP binds to the regulatory R subunits eliciting the dissociation of the catalytic C subunits. The monomeric C subunits thus released are no longer restrained catalytically and are, therefore, switched into an active conformation (Fig. 1). In contrast, the tetrameric conformation of CK2 is inordinately stable. For example, chromatography of the enzyme in presence of 1M NaCl does not disrupt the tetramer, suggesting that hydrophobic interactions mediate tetramer formation. In contrast, at sub-physiological ionic strengths (50-100 mM NaCl), the enzyme undergoes oligomerization and filament formation (Glover, 1986). The function of filament formation remains unknown, but has recently been proposed to cause downregulation of CK2 (Poole et al., 2005). A substantial search for a ligand that might elicit similar conformational transitions (akin to PKA) in the CK2 tetramer were futile. In a remarkably simple approach, Claude Cochet and Edward Chambaz resolved a tetramer of CK2 into its two subunits via SDS-PAGE. Renaturation of these SDS resolved polypeptides demonstrated that the isolated monomer of CK2α is catalytically active, albeit at levels 20% of those seen with the native tetramer (Cochet and Chambaz, 1983). Furthermore, the addition of renatured CK2β elicited a 5-fold enhancement of catalytic activity; full reconstitution of activity was achieved at a molar ratio of 1:1 for CK2β and CK2α. This remarkably led to the reconstitution of the tetrameric holoenzyme. These results have essentially been confirmed using recombinant subunits expressed in insect cells, bacteria, or yeast (Bidwai et al., 1992; Birnbaum et al., 1992; Boldyreff et al., 1993; Lin et al., 1991). Together, these results indicate that even if a ligand that could trigger a dissociation of the holoenzyme existed in vivo, at a maximum, it could achieve no greater than a 5-fold attenuation of CK2 activity. An alternative possibility of these studies is that CK2α is a non-specific kinase, specificity being conferred by CK2β. This, however, is not the case because the purified CK2α monomer displays substrate specificity virtually identical to that seen with the holoenzyme (Bidwai et al., 1993). These properties of CK2 are in stark contrast with those of PKA or CDK’s (Fig. 1). In the case of PKA, dissociation serves to activate whereas
in CDK’s the binding of the transiently synthesized cyclin engenders conformational changes that elicit activation (Draetta, 1990).

As mentioned above, the interaction of protein kinases with their substrate is highly specific and is mediated by the recognition of a specific consensus sequence in the target protein(s). The laboratory of Edwin Krebs identified such a CK2 recognition consensus using peptides with various combinations of amino acids. Based on this analysis, they proposed that the CK2 consensus is (S/T)-(D/E)-(X)-(D/E); the acidic residues at the n+1 and n+3 being rate limiting for phosphorylation (Kuenzel and Krebs, 1985; Kuenzel et al., 1987). This consensus reveals that CK2 preferentially phosphorylates Ser/Thr in an acidic microdomain. Among the Ser/Thr kinase family this property, so far, appears unique to CK2. The basic determinants of the CK2 recognition site have now been expanded upon by analyses of the phosphorylation sites in a variety of proteins that are phosphorylated by this enzyme in vitro. Some of these substrates do not meet the precise consensus mentioned above, but nevertheless exhibit a bias towards acidic microdomains (Meggio and Pinna, 2003). For example, additional acidic residues at the N- and C-terminus of this consensus only serve to further enhance phosphorylation. In addition, the effect of these acidic residues can be biochemically mimicked by phosphoserine/phosphothreonine, thus raising the possibility that CK2 can function as part of a hierarchical phosphorylation cascade/s. Such hierarchy could involve the activities of other Ser/Thr kinases or CK2 by itself. In either case this can result in multisite phosphorylation of a target protein. An example of the former is the phosphorylation of Glycogen Synthase by GSK3, CK1 and CK2 (DePaoli-Roach, 1984; DePaoli-Roach et al., 1981; Singh and Huang, 1985). An extreme example of the latter is the phosphorylation of the nuclear/cytoplasmic transport protein, Nopp140, which is phosphorylated by CK2 at 70 Ser/Thr residues in a hierarchical fashion (Meier and Blobel, 1992).

2.2. CK2 and Cell Biology:

Prior to the advent of molecular/genetic approaches, the tried and tested approaches of cell biology and biochemistry were applied to provide a glimpse into the functions of
Figure 1. Holoenzyme structure and activity comparison of CK2 with PKA and CDK.

While PKA and CDK exhibit a tight control on activity in the holoenzyme conformation, CK2α is active as a monomer as well as in the holoenzyme conformation.
CK2. A key observation that CK2 participates in the process of cell division was uncovered by the finding that levels of CK2 activity oscillated following stimulation of mammalian cells (in culture) with peptide growth hormones such as Epidermal Growth Factor (EGF) (Sommercorn et al., 1987). These oscillations occurred in synchrony with the G1/S and G2/M phases of the cell cycle. Interestingly, some of the early effectors of cell cycle progression such as Myc, Myb, Fos, and Jun were known to be phosphorylated by CK2 (Luscher et al., 1990; Luscher et al., 1989), perhaps, providing a tangible connection between phosphorylations catalyzed by this enzyme and the process of cell division. In the case of Myc and Myb, alterations of the CK2 sites have been linked to oncogenic transformations. A remarkable convergence of animal disease, epidemiology and statistics uncovered a naturally occurring biological process that shed light on the role of CK2 in cell proliferation. The laboratory of ole-MoiYoi uncovered the molecular basis of a fatal bovine disease that was characterized by classical histopathological symptoms of leukemia, i.e., hyperproliferation of B and T lymphocytes. He found that *Theileria parva*, the causative agent of Theileriosis, elicited this pathology via the overproduction and secretion of CK2 during its intralymphocytic stage of infection (ole-MoiYoi, 1995; ole-MoiYoi et al., 1993). Remarkably, the malignancy was completely reversed by treatment of infected cattle with anti-parasitic drugs. To our knowledge, this is the only case of a parasite-induced leukemia. These results were essentially corroborated by the laboratory of Philip Leder who demonstrated that mis/overexpression of CK2 under a T/B-cell promoter elicited leukemic transformation in a cell type specific manner (Seldin and Leder, 1995). Furthermore, coexpression of the proto-oncogenes Myc or Myb elicited acute lymphocytic and lymphoblastic leukemias (Kelliher et al., 1996).

Following these early findings, substantial efforts were devoted to the identification and characterization of targets of CK2. These efforts involved in vitro phosphorylation, mapping of the phosphorylation sites, and when possible effects on the functions of target proteins. Some of the targets identified during the course of these studies were, elf3, HMG17, GSK, DNA Topoisomerase II, etc. (reviewed in Glover, 1998). One aspect of CK2 that was reinforced during these studies was the acidic characteristic of its consensus recognition site. With the availability of genome sequences it became possible to predict, at a global level, all proteins in a species that harbored this consensus. Taking into account the
preference of CK2 for microacidic domains, generally thought to be solvent accessible, a tangible prediction could thus be made of a full repertoire of CK2 targets. A staggering number of potential targets of CK2 were predicted by such an analysis, leading to the suggestion that at least 10% of the cellular ‘phosphoproteome’ reflects the activity of this enzyme (Meggio and Pinna, 2003; Pinna, 2002). These ranged in function from DNA replication, transcription, translation, cell cycle progression, cytoskeletal architecture, cell polarity, development, etc. Notably underrepresented in this list are enzymes involved in intermediary metabolism, leading to the proposal by Lorenzo Pinna that CK2 performs “higher order” cell functions (Meggio and Pinna, 2003).

2.3. Molecular biology of CK2 subunits:

Once the basic biochemical properties had been established, investigators focused on elucidating the biological functions of CK2. The first step towards this goal was to identify and isolate the cDNAs/genes that encode CK2 subunits. The first indication that CK2 is highly conserved was the observation that antibodies raised against bovine CK2 cross-reacted strongly with CK2 purified from either insects (Drosophila) or mammals (humans). Using these antibodies, the laboratory of Claiborne Glover described the first successful cloning and sequencing of cDNAs encoding CK2α and CK2β (Saxena et al., 1987). The availability of these cDNA sequences led to a flurry of successful efforts on the cloning and sequencing of cDNAs and genes encoding the subunits of CK2 from cows, humans, worms, etc. Comparisons of CK2 subunit sequences from various taxa revealed an extreme level of conservation. An alignment of these protein sequences is shown in Figures 2 (CK2α) and 3 (CK2β). For example, the human CK2α subunit is 90% identical to Drosophila CK2α, while it is 98% identical to Chicken CK2α (Padmanabha et al., 1990; Saxena et al., 1987; Wirkner et al., 1994). In contrast, the yeast CK2 subunits exhibit the greatest level of divergence and are at most ~67% identical to their metazoan counterparts (Fig. 2). The regulatory β subunits show an even higher level of conservation (Fig. 3 and Bidwai et al., 1999). Expectedly, CK2α subunits display homology to other Ser/Thr protein kinases, conforming to the proposal of Tony Hunter that all of these enzymes are members of one superfamily (Hunter, 1987). In contrast, CK2β subunits do not display homology to any other proteins, apart from
**Figure 2.** Alignment of the catalytic subunits of CK2 from metazoans.

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The sequence alignments were done using the multiple sequence alignment software (ClustalW) and formatted using the Box shade algorithm. Dm-Drosophila melanogaster, Xl-Xenopus laevis, Gg-Gallus gallus, Hs- Homo sapiens. Identical residues are indicated in Red, conserved residues are indicated in Blue and nonhomologous residues are indicated in Black.
The sequence alignments were conducted as described in Fig. 2. Dm- *Drosophila melanogaster*, Xi- *Xenopus laevis*, Gg- *Gallus gallus*, Hs- *Homo sapiens*. Various conserved domains are indicated.
their homologs in those organisms. Nevertheless, the alignment of the regulatory CK2β subunits illustrates several conserved features in the primary structure that predicate some of the biochemical properties of the enzyme. Prominent among these are, the N-terminal autophosphorylation site (serine residues at position 2, 3 and possibly 4 serving as acceptors for phosphorylation), a stretch of acidic amino acids that is thought to mediate the effects of polybasic effectors, a zinc-finger motif that mediates formation of the β-β dimer, and the C-terminal residues that mediate its interaction with CK2α.

2.4. Structure of the CK2 Holoenzyme:

The extreme conservation of both CK2 subunits throughout evolution supports the notion that the fundamental properties of CK2 are unlikely to be organism-specific. As mentioned above, CK2 from various organisms exhibits the same α2β2 quaternary structure. In order to understand the precise arrangement of subunits in the holoenzyme, studies to describe the three-dimensional structure of CK2 were conducted. To date, X-ray crystallographic studies have been conducted for the isolated catalytic subunit, the regulatory β subunit dimer, and the holoenzyme (Chantalat et al., 1999; Niefind et al., 2001; Niefind et al., 1998; Rasmussen et al., 2005). These studies demonstrate that the holoenzyme is assembled by the addition of two CK2α subunits onto a β dimer. The β dimer is held together, primarily, by an interaction between the Zn finger domains of the two subunits, while hydrophobic interactions, inter subunit salt bridges and hydrogen bonds further contribute to its stability (Chantalat et al., 1999; Niefind et al., 2001). The crystallographic studies demonstrate that the two catalytic subunits in the holoenzyme do not contact each other, and the catalytic site face outwards (Fig. 4, Niefind et al., 2001). The active sites of CK2α do not make any contact with the N-terminal autophosphorylation domain of CK2β within the holoenzyme. This structural arrangement suggests that autophosphorylation is occurring in an inter-holoenzyme manner. This is in contrast to other Ser/Thr kinases that typically autophosphorylate intramolecularly. The positive role for CK2β is suggested to be due to the fact that the β subunit interacts with the N-terminal lobe of CK2α thus stabilizing it in an active conformation (Niefind et al., 2001).
Figure 4. Structure of CK2 holoenzyme.

Ribbon diagram of CK2 holoenzyme based on co-ordinates from the X-ray crystallographic data. The two regulatory subunits (yellow and blue) form the β–β dimeric core, while the two catalytic (Magenta) subunits are attached to the core with their catalytic sites facing outwards. (Adapted from Niefind et al., 2001).
2.5. Molecular-Genetic studies of CK2 functions:

The identification of cDNAs/genes thus permitted, for the first time, the generation of mutations (null or conditional) in different model organisms to better define the in vivo functions of CK2. In this regards, although cDNAs/genes encoding *Drosophila* CK2α and CK2β were the first to be characterized, it was the yeast *Saccharomyces cerevisiae* that provided the organism in which in vivo functions of CK2 subunits were first described via the genetic route. For the sake of brevity only the analysis in yeast and Drosophila is discussed.

*Saccharomyces cerevisiae*

As mentioned previously, CK2 from *Saccharomyces cerevisiae* is composed of two distinct catalytic (α and α’) and regulatory (β and β’) subunits. The catalytic subunits are encoded by the *CKA1* (α) and *CKA2* (α’) genes, while the regulatory subunits are encoded by *CKB1* (β) and *CKB2* (β’) genes (Bidwai et al., 1994; Bidwai et al., 1995; Chen-Wu et al., 1988; Padmanabha et al., 1990; Reed et al., 1994). One of the key advantage of yeast is the ability to create null alleles for a gene of interest by disrupting the endogenous copy of the gene and assessing its phenotypic consequences. This is typically achieved in a diploid strain generating a heterozygous condition. Sporulation of this diploid results in four haploid progeny. An essential gene is identified when these four haploid progeny display a ratio of 2:2 (viable:inviable). In the event that the gene is cell autonomous and recessive lethal, conditional alleles such as temperature sensitive (TS) alleles can easily be identified and employed to better define its functions.

Using the aforementioned genetic approaches, the laboratory of Claiborne Glover demonstrated that deletion of the gene encoding either catalytic subunit had no overt phenotype, raising the possibility of functional redundancy. However, the simultaneous deletion of both genes (*CKA1* and *CKA2*) is lethal (Padmanabha et al., 1990). Yeast strains harboring such double deletions undergo 2-3 cell divisions and then arrest as pseudomycelial cells that are inviable. This pseudomycelial phenotype is indicative of an uncoupling of growth and cell division, and is a hallmark of defects in cell cycle progression. To more precisely define the functions of CK2, the laboratory of Claiborne Glover also isolated
conditional (TS) alleles of the \textit{CKA1} and \textit{CKA2} genes, and used these to assess for functions. They found that temperature sensitive alleles of the \textit{CKA2} gene arrested at the G1/S and G2/M transition points, whereas those for \textit{CKA1} arrested due to defects in cell polarity. Together, these results suggest distinct in vivo roles for CK2\(_\alpha\) subunits (Hanna et al., 1995; Rethinaswamy et al., 1998). Interestingly, null alleles of CK2 in the fission yeast \textit{Schizosaccharomyces pombe} also undergo cell-cycle arrest due to cytoskeletal defects (Snell and Nurse, 1994). Given the highly conserved nature of CK2, it was not surprising that the lethality due to loss of CK2 activity could be rescued by expression of the catalytic subunit of Drosophila melanogaster CK2 (Bidwai et al., 1992). Similar efforts were also carried out with the regulatory subunit CK2\(_\beta\). Deletion of the genes \textit{CKB1} and \textit{CKB2} encoding the regulatory subunits had no effect on viability per se (Bidwai et al., 1995; Reed et al., 1994). However, strains harboring deletions of either gene rendered cells incapable of growth on media containing Na\(^+\) and Li\(^+\) (Bidwai et al., 1995). Interestingly, a yeast strain harboring disruption of both, \textit{CKB1} and \textit{CKB2}, genes does not exhibit a further increase in the level of sensitivity to Na\(^+\)/Li\(^+\). While salt sensitivity due to deletion of \textit{CKB1} can be rescued by overexpression of \textit{CKB2} the converse is not true. Taken together, these results indicate that both the subunits may perform non-redundant functions. As is the case with the catalytic subunit deletions, expression of Drosophila regulatory subunit (\(\beta\)) suppress, albeit partially, the CK2\(_\beta\) mutant phenotype (Bidwai et al., 1995).

\textbf{Drosophila melanogaster}

In \textit{Drosophila}, CK2 is composed of a single isoform of the catalytic and regulatory subunit. The catalytic subunit of CK2 is encoded by the \textit{CK2\(\alpha\)} gene located on the third chromosome (Saxena et al., 1987), while the \textit{CK2\(\beta\)} gene localizes to the X chromosome (Bidwai et al., 2000). It is important to note that the genome also harbors three additional genes that encode CK2\(_\beta\)-like proteins, whose relevance to CK2 remain unclear. These are the \(\beta\)' and SSL genes located on chromosome II and the X-linked array of Stellate (Ste) genes that are present at \(~\)100 copies (Bidwai et al., 1999; Kalmykova et al., 1997; Livak, 1990). In the case of Ste, they are normally silent in XY males due to the presence of the Y-linked Su(Ste) repeats (Balakireva et al., 1992), which represent the first example of a naturally occurring RNAi mechanism. In an XO male, one lacking the Y chromosome and
its Su(Ste) repeats, Ste undergoes derepression and expresses the protein at levels sufficient to generate crystals in primary spermatocytes (Bozzetti et al., 1995). As a result, sterility is manifest in XO males, and it has been proposed that under these conditions the Ste protein interferes with CK2 functions. The situation is further complicated by alternative transcription of the X-linked CK2β gene (Bidwai et al., 2000). On one hand, this leads to the generation of multiple transcripts with unique 5’ non-translated exons, while on the other hand it leads to transcripts that give rise to β isoforms with distinct C-termini (Bidwai et al., 2000; Jauch et al., 2002). The physiological significance/function of these β variants (mRNA’s/proteins) is unknown. It is, however, thought that the X-linked β is the predominant form in the holoenzyme based on the biochemical and immunological characteristics of the purified enzyme (Bidwai, 2000).

A screen conducted by the laboratory of Ravi Allada (Northwestern) to identify novel components of the circadian clock, led to isolation of a mutation (in CK2α) that they named as Timekeeper (Tik) reflecting its lengthened circadian period (Lin et al., 2002). Sequencing of the Tik allele demonstrates two substitutions, M161K and E165D, in residues that are highly conserved in all isoforms of CK2α (Fig. 2). The Met161 residue lies within the ATP binding pocket of the catalytic subunit, and the Lys substitution renders the enzyme catalytically inactive. In a heterozygous condition these flies exhibit only the circadian phenotype. In line with Tik encoding a catalytically dead kinase, Tik is lethal in a homozygous condition. The lethality is manifest at the first larval stage, indicating that enough maternal contribution of mRNA or protein exists to complete embryogenesis. Consistent with this, Tik displays maternal effects (Ravi Allada, personal communication). During these studies, a spontaneous revertant, called TikR, was identified based on a wild type circadian rhythm (Lin et al., 2002). Analysis of TikR revealed that this allele harbors the original two mutations seen in Tik (M161K and E165D), but in addition harbors secondary mutations that delete seven amino acids (Δ234-240) and a substitute Arg242 with Asp (Fig. 2). Predictably, TikR is also catalytically dead, and TikR/TikR is also larval lethal. The TikR protein appears to be predominantly insoluble when expressed and purified, suggesting that TikR has severe defects in folding. The revertant phenotype of TikR flies could thus be
attributed to exclusion of the mutated protein from the tetrameric holoenzyme, whereas Tik is able to ‘poison’ the endogenous enzyme.

In case of CK2β, a variety of mutations, from a simple substitution to complete gene disruptions due to the insertion of a mobile genetic (transposable) element in the gene have been isolated. The former is represented by andante, a mutant with circadian defects originally identified by Ron Konopka, in 1976. It has now been shown by the laboratory of Rob Jackson that the andante allele harbors a single nucleotide change in the CK2β gene that leads to a substitution of Met166 with Ile (Akten et al., 2003). This substitution appears to weakly predispose CK2β for degradation. Given the recent demonstration that Andante protein is fully competent to generate the holoenzyme, raises the possibility that andante may destabilize the holoenzyme itself. andante flies are homozygous viable suggesting that it is a hypomorphic allele rather than a null.

An insertion of a transposable element (P-element) in the 5’ UTR of CK2β, called DmCK2β\textsuperscript{mbup\textdagger}, was isolated during a histological screen for mushroom body defects by Tomas Rabbe’s laboratory. DmCK2β\textsuperscript{mbup\textdagger} leads to the formation of undersized mushroom bodies in the brain (due to attenuated proliferation of Kenyon cells) and is viable in a homozygous condition (Jauch et al., 2002). Imprecise excision of this P-element generated a small deletion in CK2β, called DmCK2β\textsuperscript{mbuΔA26L}, which is homozygous lethal (Jauch et al., 2002). The lethality of DmCK2β\textsuperscript{mbuΔA26L} can be rescued by expression of either the CK2β gene or by expression of a CK2β cDNA in a ubiquitous manner. The observation that lethality associated with the null allele of CK2β can be rescued by the expression of transgenic CK2β enabled a structure function analysis of different domains conserved in the regulatory subunit (Jauch et al., 2002). Such an analysis revealed that CK2β variants harboring the deletion of either the N-terminal ‘autophosphorylation’ domain, or the Zn-finger (dimerization) domain are incapable of rescuing the lethality. These results provide unambiguous evidence in support of the notion that holoenzyme formation mediated by the Zn-finger domain is critical for CK2 functions as is the autophosphorylation of the regulatory β subunit, but the precise in vivo functions of these aspects of CK2 still remain unknown.
As mentioned above, targets of CK2 are involved in a variety of processes from cell autonomous functions to development. An example of the former is DNA Topoisomerase II, whose activity is regulated by CK2 (Ackerman et al., 1985; Ackerman et al., 1988), and whose loss of function elicits lethality. Examples of the latter are Antennapedia (Antp) (Jaffe et al., 1997), Engrailed (En) (Bourbon et al., 1995), and Cactus (the homolog of mammalian IkB) (Liu et al., 1997; Packman et al., 1997). CK2 activity regulates the homeotic functions of Antp and the transcriptional functions of En. The pleiotropic functions of CK2 raise the improbability of successfully identifying development-specific alleles of this kinase. To circumvent this problem an alternative approach, and one we have used, is ‘reverse genetics’. This approach enables the analysis of the role of phosphorylation by CK2 in the functions of individual protein, without the need to perturb the activity of this enzyme per se (see below).

3. Analysis of CK2 in Drosophila melanogaster:

In the early 1990’s, molecular/genetic approaches employed in the budding and fission yeast established the roles played by CK2 during cell cycle progression, check point control and cell polarity, etc. While immensely informative with respect to cell autonomous functions of this enzyme, these studies were limited in their ability to provide any insights in the possible functions of CK2 in metazoan organisms, specifically animal development. In this regard, it is important to note the contributions of various metazoan models that have been used to study development. These include worms, fruit flies, frog, chicken, mice, etc. Of these, Drosophila provides an ideal model system to study the roles of CK2 during development given the long history of scientific analyses in this organism, and the realization that its biology, developmental programs, and signal transduction pathways are highly relevant to mammals.

3.1. Drosophila as a model system:

Adapted by geneticists in early 1900’s, studies on Drosophila melanogaster have led to a number of landmark discoveries. Thomas Hunt Morgan was awarded the Nobel Prize in 1923 for demonstrating the role of the chromosome as the basis of heredity using a naturally occurring eye color mutation in Drosophila that ultimately led to the ‘gene theory’.
Subsequently, Herman Muller (Nobel Prize in 1946), using Drosophila, demonstrated the mutagenic properties of X rays and the harmful effects of such radiations, a discovery with widespread and fundamental implications to human health. The application of Drosophila as a model system to study development did not receive attention until the astonishing discovery of the ultrabithorax (Ubx) mutations by Edward B. Lewis. These mutations elicited a complete transformation of one body part with another; specifically the ubx mutation gives rise to two thoracic segments each exhibiting a complete duplication of internal and external components. His work on Ubx mutants revealed for the first time, the presence of specific set of genes, now referred to as the homeobox. The homeobox genes determine the basic body plan of organisms during early development. In 1980, the systematic and thorough study of development in Drosophila realized its potential when Christiane Nusslein-Volhard and Eric Weischaus published their studies on the genetic controls during early embryonic development (Nusslein-Volhard and Weischaus, 1980). They used a systematic saturation mutagenesis approach to identify recessive-lethal genes that controlled virtually all aspects of early embryonic development in Drosophila. The pioneering discoveries of E. B. Lewis, Nusslein Volhard and Eric Weischaus (Nobel prize in 1995) have since been validated in other organisms, and have been instrumental in establishing the foundation for our current understanding of the genetic circuits that control virtually every aspect of development.

In recent years the ability to modify specific genes using a variety of molecular techniques, aided by the complete sequencing of the Drosophila genome, has led to the generation of a treasure trove of resources. These include, an ever-expanding collection of mutants (now maintained by Bloomington Stock Center at Indiana University), transgenics Drosophila strains, strains harboring the reporter LacZ for marking gene expression patterns, cDNA arrays and a variety of polyclonal and monoclonal antibodies (freely distributed by the Developmental Hybridoma Studies Bank, University of Iowa) that can be used to identify cell fates during development (Matthews et al., 2005). Aside from these immense resources, a unique advantage of Drosophila is its life cycle. For example, Drosophila has a short life cycle with an embryo developing into a mature adult within a span of 10 days. In addition, Drosophila is a holometabolic insect. The embryo first develops into a larva that molts three times and then reaches the pupal stage. At the pupal stage, a massive reorganization and
tissue morphogenesis is initiated to drive development into a fully mature adult. This morphogenesis presents a unique advantage over other model organisms used to study development, which has established the versatility of Drosophila as a model. In the larval body, small packets of cells called imaginal discs are set aside. During the first two larval stages, these tissues proliferate but are largely refractory to any differentiation signals until the late third larval stage. Each of these imaginal discs represents a pluripotent stem cell population that gives rise to a specific adult structure such as eyes, legs, gonads, etc. In the late third instar larvae, various developmental signals initiate the differentiation of these cells, a process that continues all the way through pupation. Thus the diversity of molecular signals and the cellular mechanism that mediate differentiation of these pluripotent cells into adult organs can be characterized by analyzing the fates of different cell types in the imaginal discs through the use of molecular markers that serve to unambiguously identify their developmental status.

3.2. Strategies to study CK2 in Drosophila:

Drosophila offers a range of potent techniques to analyze the functions of any gene of interest. These are forward and reverse genetics, and both of these approaches have been employed to study functions of CK2. A classical approach is to isolate mutations in the genes encoding CK2 subunits, and use these mutations to study the role of this enzyme during development. However, the cell autonomous functions of CK2 raise the improbability that viable mutations in genes encoding CK2 subunits can be identified, which alter its functions leading to a precise phenotypic defect during a specific developmental stage. An alternative is to use the ‘reverse genetic’ route. In this approach, CK2 interacting proteins are identified by the yeast two-hybrid approach. Following this identification, the sites of phosphorylation and domains mediating interaction with CK2 are mapped by proteomic/molecular approaches. Following this, the phosphoacceptor(s) (Ser/Thr) are modified to generate ‘loss- or gain’ of function alleles by changing them to Ala or Asp. The Asp replacement often mimics the constitutively phosphorylated Ser/Thr by virtue of the β-carboxyl group of Asp, and in the case of many Ser/Thr kinases elicits a ‘gain of function’ behavior. These alleles can then be expressed in a targeted/controlled manner. One of the earlier methods to achieve control over transgene expression was the use of a heat shock
promoter. However, this promoter elicits base line expression of the transgene even in absence of the heat shock. A tight control over transgene expression is often critical, as ubiquitous expression of a transgene interferes with normal physiology and affects viability. This issue can be addressed by the binary Gal4-UAS approach developed by Norbert Perrimon (Fig. 5, Brand and Perrimon, 1993; Duffy, 2002). This system is based on an important observation that expression of the yeast transcriptional activator Gal4 is phenotypically silent in flies presumably due to the absence of binding sites. Thus transgenes are engineered with an Upstream Activating Sequence (UAS) that allows for Gal4 binding. The expression of such a transgene is achieved only when flies harboring the UAS-transgene are mated with those expressing the yeast Gal4 protein (Fig. 5). A large collection of fly stocks have been generated each expressing the yeast Gal4 protein in a specific tissue or at a specific time during the development. These stocks were generated by the random insertion of a promoter-less Gal4 cassette. If the insertion of this cassette occurs downstream of a temporal or tissue-specific enhancer, Gal4 is produced in a manner that mirrors that of the endogenous gene. The Gal4-UAS system has been widely used to analyze the functions of various genes, and one of the most notable examples of its success is the generation of 'ectopic' eyes upon expression of the mammalian Pax6 protein (Halder et al., 1995).

3.3. CK2 as a potential regulator of bHLH repressors during neurogenesis:

A large-scale effort to identify CK2 interacting proteins by the two-hybrid approach was conducted by our laboratory (Trott et al., 2001a). This screen led to the identification and isolation of a large pool of CK2 interacting proteins, many of which appear to represent potential targets for phosphorylation by the kinase. Notable among these are proteins such as SSL, E(spl)M7, TFIIIA, and Spalt. In this section, I restrict my discussion to the interaction of CK2 with E(spl)M7.

E(spl)-M7 is a terminal effector of the Notch signaling pathway (see below) and is encoded by the Enhancer of Split Complex, E(spl)C. The E(spl)C locus encodes a group of highly conserved basic-Helix-Loop-Helix (bHLH) proteins (Mα, Mβ, Mγ, M3, M5, M7, and M8) and the non-bHLH proteins, M4 and Groucho (Fig. 6A, (Delidakis and Artavanis-Tsakonas, 1991; Delidakis et al., 1991; Knust et al., 1992) ). Given the overall structural
Figure 5. The bipartite Gal4-UAS system in Drosophila (adapted from Duffy., 2002).

When flies carrying the UAS-transgene are mated those carrying a GAL4 driver progeny containing both the elements are produced. The presence of a Gal4 protein drives the expression of the UAS-transgene. 

B and C exemplify the use of Gal4-UAS system. Human Pax6 gene is overexpressed in flies and leads to induction of ectopic eyes.
similarities of the bHLH members, our laboratory sought to test whether the CK2-M7 interaction was specific to this bHLH protein, or could also be observed with other members of this complex. Explicit tests for interaction of CK2α with other E(spl) members were thus carried out to address this question. This analysis indicated that interaction was not restricted to M7, but CK2 interacts with M5 and M8 as well. The specificity of interaction between CK2 and these bHLH members was found to correlate to the presence of a highly conserved phosphorylation site in the C-terminal region of these three members (Fig. 6C), while none of the non-interacting members showed the presence of the phosphorylation consensus (Trott et al., 2001b). Consistent with this observation, CK2 was found to specifically phosphorylate these three bHLH repressors (recombinant) in an in vitro assay employing purified CK2. Surprisingly, it was found that Drosophila CK2α either as a monomer (purified from yeast expression system) or the Drosophila embryo holoenzyme, could equivalently phosphorylate these three bHLH proteins raising the possibility that the regulatory β subunit does not modify the interaction/phosphorylation. As mentioned above, E(spl) bHLH members function to restrict the number of cells assuming the neuronal cell fate in the Notch signaling pathways. The observation that CK2 can phosphorylate these E(spl) bHLH proteins is therefore significant as this was the first evidence implicating this kinase as a regulator of Notch signaling.

3.4. Notch signaling and the role of Enhancer of Split repressors:

Notch signaling is involved in specification of numerous cell fates during diverse developmental processes (for reviews, see Blaumuller and Artavanis-Tsakonas, 1997; Lai, 2002; Mumm and Kopan, 2000). Examples of its varied developmental roles are the process of neurogenesis, myogenesis, oogenesis, and the specification of secretory cell fate. I restrict my discussion to its roles during the process of neurogenesis, which in Drosophila is best studied with respect to the development of mechanosensory bristles and the eye.

This signaling pathway consists of the transmembrane receptor Notch (N), and other loci such as Delta (Dl), Serrate (Ser), Suppressor of Hairless (Su(H)), and the Enhancer of Split (E(spl)), to name a few. Among these, Dl and Ser encode ligands of Notch, Su(H) encodes a transcription factor, and E(spl)C the ultimate target of Notch signaling. As
Figure 6. The Enhancer of the split Complex.

A. The Enhancer of the split Complex.

B. Functional Domains of Enhancer of split M8.

C. Conserved CK2 site in M5, M7 and M8.

A. Genes of the Enhancer of split Complex are arranged in a cluster on third chromosome (96F8-96F10) in Drosophila melanogaster. B. Functional domains of E(spl)M8, bHLH (basic-DNA binding and Helix loop Helix-dimerization), Orange (interaction with specific proneural proteins), CK2 phosphorylation site and the C-terminal groucho interaction domain. The numbers indicate the amino acid residues. C. Shows the conserved CK2 phosphorylation site in M5, M7 and M8 proteins, the phosphoacceptor Serine is marked red.
Figure 7. Notch mediated Lateral Inhibition.

**A. Lateral inhibition**

![Diagram showing lateral inhibition](image)

**B. Notch signaling during lateral inhibition.**

![Diagram showing Notch signaling](image)

**A.** The number and pattern of the Sensory Organ Precursor (SOP) cells is laid down by 'lateral inhibition'. A single SOP cell, develops from a population of equivalent neuronal competent cells (Red). The SOP inhibits surrounding cells from taking the Neuronal cell fate. These cells then take up alternative cell fate (green).

**B.** Representation of the important steps of Notch signaling, in Drosophila. The mammalian homologs are indicated in parentheses. In Drosophila and mammals, the expression of E(spl)C bHLH and the HES protein respectively, leads to repression of neuronal cell fate.
mentioned above, the \(E(spl)\) family encodes for seven bHLH proteins. \(E(spl)\) expression is induced by the Notch signaling pathway. An interaction between the ligand Delta and the receptor Notch leads to activation of the Notch signaling cascade (Fig. 7A). The activated Notch protein undergoes a cleavage to release a Notch Intra-Cellular Domain (\(N^{ICD}\)). The \(N^{ICD}\) then associates with Su(H) converting it from a repressor to an activator. This leads to transcription of \(E(spl)\) proteins that function as repressors of neurogenesis (reviewed in Artavanis-Tsakonas et al., 1995). All the \(E(spl)\) bHLH proteins mediate repression upon recruitment of Groucho (a co-repressor) (Chen and Courey, 2000; Delidakis and Artavanis-Tsakonas, 1991; Fisher and Caudy, 1998; Jimenez et al., 1997; Paroush et al., 1994).

At a structural level, \(E(spl)\)-bHLH repressors have multiple functional domains. These include a basic domain (that mediates DNA-binding), an helix-loop-helix domain (that mediates homo/hetero dimerization amongst \(E(spl)\) members), an Orange-domain (that mediates the specificity of their interaction/s with proneural bHLH proteins) (Dawson et al., 1995; Gigliani et al., 1996), and an invariant C-terminal tetrapeptide, WRPW, that mediates recruitment of Groucho (a protein containing Transducin-like repeats) which is necessary for transcription repression (Fig. 6B). It is noteworthy that a large family of similar proteins exists in mammals. These include homologs of the \(E(spl)\) bHLH proteins that are collectively referred to as HES (Hairy Enhancer of Split like) proteins (Ishibashi et al., 1994). In addition, Groucho also has homologs in mammals, and these are referred to as the TLE’s (Transducin-like effectors) (Chen and Courey, 2000). In both taxa, HES-Groucho complexes regulate cell-fate specification in response to Notch signaling.

3.5. The role of Notch during Neurogenesis:

This discussion is restricted to the roles of Notch signaling during the process of neural cell-fate determination, which occurs in the neuroectoderm. In the neuroectoderm the neuronal cell-fate is the default pathway. If allowed to proceed in an unrestricted fashion, this pathway would result in supernumerary neurons. This, in turn, would lead to a deficit of other supporting cell types thus causing an interference with proper function of a particular sense organ such as the eye or the mechanosensory bristle (see below). During development of the nervous system, the cells that are destined to take up the neuronal cell fate signal to the surrounding cells and inhibits them from taking up an identical cell-fate. This has been
termed 'lateral inhibition' a process that appears to be exclusively mediated by cell-cell communication in a localized context (Fig. 7A, B). Lateral inhibition is mediated by Notch signaling, thus restricting the number of neuronal precursors and allowing a precision of patterning that is necessary for proper organogenesis and/or histogenesis (Artavanis-Tsakonas et al., 1995). As stated above, the ability of Notch signaling to repress neuronal fate is mediated via expression of E(spl)-bHLH repressors. In Drosophila, the role of E(spl)-bHLH repressors, in blocking neuronal fate has been studied during formation of sensory organs such as the mechanosensory bristle and the eye (Nakao and Campos-Ortega, 1996).

3.6. Role of E(spl) repressors during bristle and eye morphogenesis:

In Drosophila, bristles constitute a major group of mechanosensory organs and are enervated by a single nerve. It should be noted that the number and positioning of these mechanosensory bristles is invariant and is largely dependent on proper Notch functions. For example, the scutellar bristle (macrochaete) develops from a single sensory organ precursor (SOP) cell that is selected from a group of equipotent cells (Pro Neural Cluster, PNC) in the wing discs of the third larval instar. In a mechanism that still remains a mystery, one of the cells of the PNC gains an advantage and is selected to go on and become ‘the’ SOP cell (Fig. 8A). This SOP then undergoes cell division to give rise to the different cell types that constitute the mechanosensory bristle (Fig. 8A). Notch regulates the selection of SOP from the PNC because upon loss of Notch, all cells of the PNC go on to adopt an SOP fate. This selected cell signals to its neighbors via Delta, the activating ligand of Notch. Cells that receive this signal express the E(spl) repressors, which then redirect their development away from the neuronal (SOP) cell fate. Consistent with this model for the role of Notch, loss of E(spl) elicits neural hyperplasia (ectopic bristles and duplicated bristles), whereas ectopic overexpression of E(spl) proteins results in a dominant suppression of bristles. The ability of the E(spl) bHLH repressors to dominantly suppress bristle formation (upon ectopic expression) has been widely used to assay for their function(s) in vivo.

The compound eye of Drosophila is composed of ~800 unit eyes called ommatidia that are arranged in a precise hexagonal two-dimensional array. Each ommatidium is composed of eight photoreceptor neurons called R-cells (R1-R8), and 12 accessory cells
A. A cluster of cells expressing proneural proteins is specified. Lateral inhibition plays role during SOP selection in the neuroectoderm (1) as well as during further differentiation wherein a single SOP differentiates into the different cell types that constitute a bristle (2). B. Retinal patterning in the eye imaginal disc of Drosophila 3rd instar larvae. Arrows indicate the movement of the morphogenetic furrow (MF) that patterns R8 formation via Atonal expression. Atonal is expressed as a broad band in the pre-furrow region (orange). In the MF Atonal expression becomes restricted to a cluster of cells (red). Posterior to the MF the cells in these clusters (green) undergo lateral inhibition through Notch signaling, except the future R8 (red).
including, cone cells (that secrete the lens), pigment cells (that optically shield each ommatidium from its neighbors), and a single mechanosensory inter ommatidial bristle. Eye morphogenesis initiates in the eye imaginal disc, a monolayer epithelium, which is subject to multiple controls to generate the diverse array of cell types. This program initiates during the third larval stage. During this process, a wave of differentiation called the morphogenetic furrow (MF) sweeps across the disc epithelium over a period of 48 hours (Fig. 8B). This movement of the MF occurs from the posterior to the anterior end of the disc. Within the MF (and behind it), cell fate specification is choreographed in a temporally and spatially ordered manner. This specification involves interactions between numerous signaling pathways such as Wnt/wingless, Notch, EGFR, etc (reviewed in Frankfort and Mardon, 2004; Hsiung and Moses, 2002). However, the diversity of cell types that comprise each ommatidium is predicated on the specification of a single neural subtype, the R8 cell. The R8 photoreceptor neuron is the first cell to be specified, reasons for which it is referred to as the ‘founding’ photoreceptor. This R8 cell then mediates recruitment of other photoreceptors (R1-R7). Because patterning of the eye is dictated by the precise spatial specification of R8’s, any perturbation of this process interferes with the hexagonal spacing and manifests as a rough eye. Such perturbations have been, and continue to be, used to assess the roles of proteins with functions encompassing cell cycle progression, cell polarity, etc.

R8 specification is dependent on Notch signaling. In an early phase termed 'proneural enhancement', Notch elicits atonal (ato) expression in 'intermediate' group cells (akin to PNC’s) and this expression serves to maintain neural competency (Fig. 9A, 9B, and Baker et al., 1996; Baonza and Freeman, 2001). Subsequently, Notch mediates refinement ('lateral inhibition') in the 'intermediate' group cells (lateral inhibition) via E(spl) expression (Fig. 9B), thus restricting ato expression to a single cell that goes on to differentiate as an R8 cell (Ligoxygakis et al., 1998). The remaining cells of the group do not adopt a neural fate, but remain uncommitted thereby ensuring their competency for subsequent recruitment (by the R8 cell) into the developing ommatidium. It is somewhat surprising, and perhaps unique to the eye, that both of these functions ('proneural enhancement' and 'lateral inhibition') affect the same cell fate decision. Consistent with this, an absence of N during 'proneural enhancement' severely reduces ato expression and results in a loss of R8 cells (as seen in ato flies). In contrast, an absence of N during 'lateral inhibition' prevents resolution of
Figure 9. Biphasic Notch signaling during eye morphogenesis.

Schematic representation showing only the critical steps during the biphasic role of Notch signaling in R8 formation. In the first phase (1), Notch induces Atonal expression anterior to the MF. In the second phase (2), it activates M8 expression and inhibits expression of the proneural Atonal.
'intermediate' groups into single phase-shifted R8 cells. In this case, as a consequence virtually all cells of the 'intermediate' group continue to express Atonal (which is subject to a positive feedback loop (Sun et al., 1998) and thus go on to adopt an R8 cell fate (Ligoxygakis et al., 1998). Predictably, eye disc clones lacking $E(spl)$ bHLH-repressors (but containing normal amounts of Groucho) are compromised only in refinement (lateral inhibition) and thus exhibit supernumerary R8 cells. Of the seven $E(spl)$ genes, three ($m8$, $mβ$, and $mδ$) are expressed in the eye disc. However, $m8$ is thought to play a predominant role in this tissue, because its overexpression and mutation in the $E(spl)D$ allele (encoding M8*) severely attenuates retinal morphogenesis, and because the loss of $mβ$ and $mδ$ does not affect eye development (Nagel and Preiss, 1999; The et al., 1997). Inspite of an immense body of evidence on the genetic circuits that mediate Notch effects during development, it has largely remained unclear if this signaling pathway is regulated by phosphorylation. The studies we have conducted suggest that CK2 provides a means whereby the repressive effects of Notch are modulated in a dynamic manner. Given the conservation of the mechanism we have uncovered to that in humans, it is likely that the studies in Drosophila will yield important insights into the developmental roles of this protein kinase.

4. Scope of dissertation:

The aim of this dissertation is to address three aspects of CK2 functions in Drosophila. The first of these is to characterize the relevance of SSL, a CK2$β$-ortholog (Chapter 2). The second is to define the role of phosphorylation of E(spl)M8 during neurogenesis (Chapters 3 and 4). The third is to characterize Deadpan, a pan neural protein, as a target of CK2 (Chapter 5). The implications of these findings are discussed in the overall conclusions.

Chapter 2.

We had identified SSL as a CK2 interacting protein from an embryonic cDNA library. This was contrary to published data arguing that SSL is a testis specific gene. I have used biochemical and molecular approaches to address the relevance of SSL to CK2. These studies suggest that SSL interacts with CK2$α$ with an affinity equivalent to that seen with
CK2β, and employs an interaction domain identical to that with CK2β. In addition, a yeast complementation assay indicates that, like CK2β, SSL also rescues the ion homeostasis defects due to a mutation in the yeast CK2β genes. Finally, I demonstrate that the expression of SSL encompasses a greater developmental window and is not testis-specific. These results were published in Biochemical and Biophysical Research Communications in 2003.

Chapter 3.

As mentioned above, analysis of the E(spl) bHLH members revealed that CK2 interacts with and phosphorylate, M5, M7 and M8. The role(s) of E(spl)-M8 have been studied during Notch mediated lateral inhibition. Previous studies (from our laboratory) indicate that M8 is phosphorylated at Ser\(^{159}\) by CK2. In this chapter the effects of phosphorylation of M8 by CK2 have been analyzed via the Gal4-UAS approach employing the nonphophorylatable (M8SA) or the constitutively phosphorylated (M8SD) variant of E(spl)M8. These variants were expressed during bristle and eye development. The results indicate that phosphorylation of M8 by CK2 is critical for its role in the selection of ‘founding R8 photoreceptor’ during eye morphogenesis, but does not appear to be critical during bristle morphogenesis. Expression of M8SD results in neural hypoplasia, and a loss of Atonal positive cells resulting in a severe reduction of the eye field. This phenotype mirrors that of E(spl)D, an allele of the m8 gene that encodes a variant lacking the CK2 phosphorylation site. Our analysis reveals that CK2 phosphorylation of M8 is essential for its ability to block the proneural protein, Atonal, needed for R8 specification, and provides a mechanism for the behavior of the E(spl)D allele. These findings along with their implications to the current understanding of R8 photoreceptor specification have been published in Mechanisms of Development in 2004.

Chapter 4.

In this chapter, the role of CK2 in the regulation of M8 has been further explored. We demonstrate that the reduced eye phenotype of M8SD is antagonized by the simultaneous overexpression of Ato or by a reduction in the dosage of CK2. In addition, we have analyzed the rough eye phenotype of M8SA, and find that this variant behaves as an antimorph. Finally, we have analyzed the in vitro and in vivo behavior of the isolated phosphorylation subdomain of the M8 protein. The potential implications of these results been discussed.
Chapter 5.

Deadpan (Dpn) a pan neural protein (Emery and Bier, 1995; Roark et al., 1995) in Drosophila shows conservation at structural level with E(spl) (Dawson et al., 1995) and is also known to function as neuronal repressor because it downregulates the expression of proneural proteins. However unlike E(spl), Dpn is expressed in the cells that have taken up the neuronal cell fate and is suggested to function to downregulate the activity of proneural proteins once the cell fate has been decided. Apart from neural development, Dpn also functions during sex determination where it mediates repression of \textit{sxl}, a master regulatory gene of this developmental pathway (Younger-Shepherd et al., 1992).

During the course of analyzing a catalytically dead CK2α subunit, we serendipitously uncovered that Dpn might represent a bonafied CK2-target. Earlier efforts to analyze the CK2-Dpn interaction by the yeast two-hybrid approach had elicited ‘weak’ induction of yeast two-hybrid reporter genes, and because these values were insignificant in relation to those with CK2-M8, it was thought to indicate a non-interaction. The use of a catalytically dead variant suggesting that another E(spl)C related neuronal repressor called Deadpan (Dpn) might be phosphorylated by CK2 (Shaffer et. al., manuscript in preparation). The possibility of phosphorylation of Dpn by CK2 was explicitly analyzed and the results of this analysis with its potential implications have been discussed in Chapter-5 and have recently been published in Molecular and Cellular Biochemistry in 2005.
Chapter 2.

The Drosophila SSL gene is expressed in larvae, pupae, and adults, exhibits sexual dimorphism, and mimics properties of the β subunit of Casein Kinase 2.
1. Abstract

*Drosophila melanogaster* casein kinase 2 (CK2) is composed of catalytic and regulatory β subunits that generate the α2β2 holoenzyme. A two hybrid screen of a Drosophila embryo library using CK2α as bait has resulted in the isolation of multiple cDNAs encoding SSL, a CK2β-like polypeptide. We demonstrate that CK2β, β', and SSL exhibit robust and comparable interaction with CK2α. Residues in SSL that mediate interaction with CK2α appear similar to those in CK2β, and SSL forms homodimers and heterodimers with CK2β or β' as well. We have tested all known Drosophila CK2β-like proteins for rescue of the ion-homeostasis defect of yeast lacking subunits, and find that CK2β and SSL complement, β' has marginal function, and Stellate appears non-functional. We have used real-time RT-PCR to assess developmental expression, and find that CK2β is robust and ubiquitous, whereas SSL is restricted to males (third-instar larvae, pupae, and adults), but is nondetectable in females of the corresponding stages. These results indicate that SSL expression encompasses a greater developmental window than previously suggested, and may confer distinct functions to CK2 in a sex-specific manner.
2. Introduction

CK2 is a highly conserved Ser/Thr protein kinase that is ubiquitous in eukaryotes (reviewed in Pinna, 2002). The enzyme is composed of catalytic (α) and regulatory (β) subunits, which associate to generate the α₂β₂ holoenzyme. With the exception of *D. melanogaster* (Saxena et al., 1987), *Caenorhabditis elegans* (Hu and Rubin, 1990), and *Schizosacharomyces pombe* (Roussou and Dretta, 1994), CK2 from most organisms contains two distinct isoforms of the catalytic subunits that are encoded by separate genes. On the other hand, β subunit heterogeneity has only been documented in *Saccharomyces cerevisiae* (Bidwai et al., 1994), *Arabidopsis thaliana* (Collinge and Walker, 1994), and *D. melanogaster* (Bidwai et al., 1999). CK2 phosphorylates Ser/Thr within hyperacidic microdomains and its consensus for phosphorylation can best be described as (S/T)(D/E) X (D/E) (Kuenzel et al., 1987). Consistent with this, a number of proteins essential for DNA-replication, transcription, translation, cell cycle regulation, and cell signaling contain such sites and are known to be phosphorylated in vitro and in vivo (Glover, 1998). The enzyme is activated by polybasic compounds such as spermine, polylysine, and protamine which mediate these effects via the β subunit, whereas polyacidic compounds such as polyglutamate are believed to inhibit activity via competition with acidic microdomains of target proteins.

Studies using recombinant proteins suggest that when compared to the holoenzyme, monomeric CK2α exhibits approximately 20% of the activity (Cochet and Chambaz, 1983). In line with this observation, CK2β stimulates activity ~5-fold and full reconstitution of activity correlates with assembly of the holoenzyme. The stimulation of activity by CK2β involves both the affinity (km) for the substrate and its rate (Vmax) of phosphorylation (Bidwai et al., 1993). Additionally, CK2β is (auto)phosphorylated at its N-terminus, MS²S²SEE, (catalyzed by CK2α) and this reaction has been suggested to mediate turnover via ubiquitination and proteosome-mediated degradation (Zhang et al., 2002). Given that CK2 activity is messenger-independent and that no regulators have been identified to date, regulation by (auto)phosphorylation may represent a potential mechanism to downregulate
CK2 in vivo. Collectively these studies suggest that the β subunit serves a critical regulatory function.

Genetic analyses in a number of model systems have begun to clarify the roles of CK2β. While loss of this subunit is dispensable for viability in yeast, it elicits severe defects in ion-homeostasis in *S. cerevisiae*, and this phenotype is rescued by expression of Drosophila CK2β (Bidwai et al., 1995), demonstrating functional conservation. CK2β is recessive lethal in Drosophila (Jauch et al., 2002) and, given the inordinately high conservation of this subunit, its essentiality may be applicable to all metazoan organisms. An hypomorphic allele of CK2β is associated with a reduction in the size of the mushroom body due to attenuation of cell proliferation and a decrease in number of Kenyon cells (Jauch et al., 2002), results that corroborate a requirement of this enzyme for cell cycle progression (Glover, 1998). Surprisingly, the lethality associated with loss of CK2β is only 'partially' rescued by a non-autophosphorylating isoform, raising the potential importance of this reaction in regulation of CK2 functions in vivo. Drosophila also harbors two autosomal genes that encode β-like proteins, i.e., β' (Bidwai et al., 1999) and SSL (Kalmykova et al., 1997), and a multicopy locus on the X-chromosome known as Stellate (Ste) (Livak, 1990). The Ste locus is potently repressed in XY males due to the Y-linked Su(Ste) locus (Balakireva et al., 1992). Consistent with this, absence of Su(Ste) in XO males elicits massive overproduction of Ste which forms crystalline aggregates in spermatocytes and elicits sterility (Bozzetti et al., 1995). It has been previously suggested that Ste may mimic CK2β (Bozzetti et al., 1995), but structure/function studies argue against this possibility because this protein appears to lack residues critical for interaction with CK2α (see below). The relevance of Ste to CK2 thus remains unclear. The functions of β' and SSL also remains enigmatic. Both proteins conserve motifs required for interaction with CK2α, but given the recessive lethality of CK2β, it is likely that these isoforms have distinct functions.

We report here the isolation of multiple cDNAs encoding SSL from an embryo two-hybrid library, and demonstrate that interaction of SSL with CK2α is robust and equivalent to that observed with CK2β or β'. Deletion analysis confirms that residues mediating the CK2α-CK2β interaction are conserved in SSL. Additionally, SSL is capable of forming homodimers as well as heterodimers with CK2β or β'. Furthermore, we demonstrate that
CK2β or SSL exhibit comparable efficiency in their ability to rescue the ion-homeostasis-defects of ckb1/2 yeast. Finally, real-time RT-PCR analysis suggests that, unlike CK2β, which is expressed throughout development, transcripts encoding SSL are readily detectable in third instar larvae, pupae, and adults in a male-specific manner. These studies suggest that SSL may confer a sexually-dimorphic modulation of CK2 functions.
3. Materials and Methods

3.1. Two-hybrid screen

The open-reading-frame encoding CK2α was amplified by PCR using primers that introduce EcoRI and BamHI sites at the 5' and 3' ends, respectively, and subcloned into the plasmid pGBT9 (Clontech) wherein the cDNA is expressed as a fusion with the DNA-binding (DB) domain of *S. cerevisiae* Gal4. The insert was sequenced on an Applied Biosystems DNA Sequencer 373A to confirm the correctness of the sequence. Yeast strain HF7c (Feilotter et al., 1994) expressing GAL4DB-CK2α fusion protein was used as the host strain to screen a 3-18 hour *Drosophila* embryo cDNA library (kindly provided by S. J. Elledge, Baylor College of Medicine, Houston). Briefly, the two-hybrid cDNA library (~1x10⁸ total recombinants) inserted in the plasmid pACT (Durfee et al., 1993) was transformed into the host strain, and an estimated 4x10⁸ transformants were plated on medium lacking Trp, Leu, and His, and colonies (~650) showing robust growth were counterscreened for expression of β-galactosidase as described (Miller, 1972). ~90 His+/LacZ+ Clones were subjected to loss of the bait-encoding plasmid, and those that also exhibited a concomitant loss of HIS3 and LacZ expression were chosen for further analysis. Library plasmids were recovered in *E. coli* and those encoding CK2β/β' were identified by PCR using specific primers. The remaining cDNAs were identified by DNA sequencing. From this analysis, 5 clones encoding SSL were recovered and their isolation forms the basis of this study.

3.2. Explicit two hybrid interactions

None of the SSL clones isolated by us contained a full-length 5' end. We, therefore, selected clone DmA90-464 (that lacks only the start codon), and full length SSL was reconstructed by PCR using SSL-specific primers that also introduce BamH1 and XhoI sites at the 5’ and 3’ ends, respectively. Constructs that express CK2α, CK2β, β’, or SSL were generated in the plasmid pACT, where proteins are expressed as fusions with the activation domain (AD) of Gal4. Deletions in SSL, i.e., SSL-G148*, SSL-E173*, and SSL-K186*, were generated using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions, and mutations were confirmed by sequencing using
dideoxy-chain terminations on an Applied Biosystems DNA sequencer 373A. Full-length SSL and truncations were combined with pACT constructs expressing CK2α, CK2β, β', or SSL, and interactions were assessed in yeast HF7C by growth in minimal media lacking histidine (His-) and by LacZ assays on two independent transformants each in triplicate.

3.3. Suppression of salt sensitivity of ckb1 or ckb2 S. cerevisiae

cDNAs encoding β' or SSL were subcloned into the plasmid, pESC-Ura (stratagene), where proteins are expressed with a Flag-epitope tag (at their C-terminus) under control of a synthetic GAL promoter. Construction of plasmids that similarly express CK2β or Stellate have been described previously (Bidwai et al., 1995). Plasmids expressing CK2β, Stellate, β', and SSL were introduced into yeast harboring a deletion of either the CKB1 (YAPB7) or CKB2 (JCR8) gene using lithium acetate (Guthrie and Fink, 1991), and transformants were selected on minimal medium lacking uracil. Subsequently, transformants were grown in rich medium containing galactose as the sole carbon source (YPGal), adjusted for cell numbers, and plated onto YPGal or YPGal+0.5M NaCl essentially as described. Plates were incubated at 29°C and photographed.

3.4. Real Time RT-PCR

Primer and probes for real-time RT-PCR of CK2β are as follows; forward primer, 5'-GTTTAAATGAGCGAGGTACCCAACTA-3'; reverse primer, 5'-TCGTCCTCCGGTTCCAAGT-3'; FAM (6-carboxyfluorescein) labeled probe that spans Exons 1 and 2 of the CK2β gene, 5'-CGCCAAGCGTTGGACATGATCTTG-3'. Primer and probes for real-time RT-PCR of SSL are as follows; forward primer, 5'-TCCGCCCGTGGAGAAGTAC-3'; reverse primer, 5'-GGACTTGCTGACCGAGGATTC-3'; FAM labeled probe that spans Exons 1 and 2 of the SSL gene, 5'-CCCGTGATATGGCTCCAGTGTCGAC-3'. Primers and probes were designed using the Primer Express software (Applied Biosystems Inc.). The optimal conditions for the primers and probes were identified on RNA isolated from pooled larvae and pupae using the High Pure RNA Isolation Kit (Roche) that was reverse transcribed using the Taqman Gold RT-PCR kit (Applied Biosystems Inc.). Based on this analysis, the optimal conditions that were used for analysis were as follows: SSL, forward primer (500nM), reverse primer
(250nM), and the FAM-labeled probe (250nM); CK2β, forward primer (200nM), reverse primer (275nM), and the FAM-labeled probe (250nM).

Male crawling third-instar-larvae were identified by the presence of testis, and RNA isolated from a single individual was used as a template. In addition, male third-instar-larvae were selected and allowed to undergo pupation, and RNA isolated from a single individual was used as a template. Females at similar stages were isolated in an analogous manner. RNA was isolated from single larvae, pupae, and adults (male and female) using the High Pure RNA Isolation Kit (Roche) that was reverse transcribed using the Taqman Gold RT-PCR kit (Applied Biosystems Inc.). Real time RT-PCR was performed using ABI’s Universal PCR master mix, and the lowest Ct value and highest ΔRT define optimal conditions for the primers and probes. A 10μL aliquot of the cDNA was added to a 40μl reaction master mix containing all of the ingredients (forward and reverse primers, probe, and master mix), and reactions containing water and the master mix were run, in parallel, as a negative controls. Real-Time RT-PCR was preformed on an ABI’s 7700 PCR machine using default parameters. Fluorescence output results were captured and analyzed using Sequence Detection Software Version 1.7 (Applied Biosystems Inc.), and the threshold cycle (Ct) was used for assessing relative levels of CK2β vs SSL transcripts.
4. Results & Discussion

4.1. Isolation of cDNAs encoding SSL from embryo libraries.

The yeast strain HF7C expressing CK2α as a bait was used to screen a D. melanogaster 0-18 hour embryo two-hybrid cDNA library. From ~4x10^8 transformants, 90 clones that activate HIS3 and LacZ in a bait-dependent manner were recovered. PCR analysis was used to eliminate clones that encode CK2β or β', since these are likely to be isolated in a two hybrid hunt at a high frequency because of the high-affinity α-β or α-β' interactions (Bidwai et al., 1995). The remaining clones were retested against Gal4DB-alone or Gal4DB-CK2α to ensure specificity, and those that induced reporter gene expression only in combination with CK2α were identified by DNA sequencing. Sequencing revealed that this screen has so far yielded 13 clones encoding rpL22 (Zhao et al., 2002), 7 encoding E(spl)m7 (Trott et al., 2001b), 5 (DmA90-35, -154, -279, -330, -464) encoding SSL, and the rest encode novel proteins that will be described elsewhere. DNA sequences of the five SSL-encoding cDNAs revealed that three clones (DmA90-35, -154, and -330) are missing the first 18 nucleotides of the open-reading-frame, whereas two (DmA90-154 and -464) are lacking the initiation codon. Additionally, none of these clones contain a poly(A)tail suggesting that they are not full-length with respect to their 3' untranslated regions.

Our isolation of cDNAs encoding SSL in a random two hybrid screen of an embryo cDNA library was surprising because it has been recently suggested that SSL is testis-specific (Kalmykova et al., 2002). The Drosophila genome harbors three genes that encode CK2β-like proteins, i.e., β' (Bidwai et al., 1999), SSL (Kalmykova et al., 1997), and Ste (Livak, 1990). Sequence alignments and phylogenetic analysis suggest that β' is more closely related to CK2β, than are SSL and Ste (Bidwai et al., 1999), and the relevance of Ste to CK2 is unclear because it does not appear to interact with CK2α. We, therefore, deferred from parallel analysis with this isoform, and focused on CK2β, β', and SSL. To provide for a comprehensive and controlled analysis, identical constructs encoding these three proteins in the Gal4AD-vector, pACT, were tested against CK2α in the Gal4DB-vector, pGBT9 (see Materials and Methods). As expected, expression of CK2α, CK2β, β', or SSL by themselves did not elicit transcription of HIS3 or LacZ (Fig. 10A). On the other hand, coexpression of
CK2α+CK2β elicited robust induction of both reporter genes, indicative of a high-affinity interaction, and in this regard β' and SSL also exhibit a comparable affinity for CK2α (Fig. 10B). The reasons underlying the higher LacZ values for the CK2α+SSL combination, compared to those for CK2α+β/β', are presently unclear, but it is worth noting that identical values are obtained when the orientation of CK2α and SSL with respect to Gal4DB or Gal4AD are reversed. Thus the SSL-CK2α interaction is orientation-independent, as is the case for interaction of CK2α with β/β' (Bidwai et al., 1999). These results are somewhat analogous to those recently obtained by Kalmykova et al., (2002), but the virtually identical induction of LacZ (Fig. 10B) would suggest that CK2β, β', and SSL exhibit a comparable and high affinity interaction with CK2α. Based on the observation that CK2β is a dimer (Chantalat et al., 1999), we have also tested this property of the SSL protein. The robust growth of cells coexpressing Gal4DB-SSL + Gal4AD-SSL fusion proteins in His- media suggests that SSL can also form homodimers, although the levels of LacZ appear significantly attenuated compared to those with SSL+CK2α (Fig. 10B). Additionally, SSL also appears to be competent at forming heterodimeric complexes with CK2β or β'. We attribute the attenuated LacZ values, not to a weak interaction, but rather to a general instability of Drosophila β-like proteins in yeast. We have previously observed that expression of CK2β in yeast does not elicit significant protein accumulation unless coexpressed with CK2α (Bidwai et al., 1992), and in line with this observation, CK2α+CK2β versus CK2β+CK2β interactions assessed via two hybrid analysis demonstrates that the latter combination exhibits a high attenuation of LacZ induction (Bidwai et al., 1999). Consistent with these observations, LacZ values are substantially higher when these β-isoforms are co-expressed with DmCK2α (Fig. 10). Taken together, these results raise the likely possibility that holoenzyme isoforms can be built with equivalent efficiencies using a homodimeric core containing β (α2β2), β' (α2β'2) or SSL (α2SSL2), and also suggest that tetramers containing distinct β-isoforms, e.g., α2ββ', α2βSSL, etc. can also be generated. The identification of substrates that interact exclusively with the α2β2 holoenzyme will be necessary to determine whether these isoforms confer distinct functions.
**Figure 10.** Isolation of SSL and interaction with DmCK2 subunits.

*S. cerevisiae* strain HF7C was transformed with plasmids expressing the indicated fusions with GAL4DB or GAL4AD. Transformants were selected, and following growth in glucose medium, cultures were tested for induction of *HIS3* expression by plating on complete minimal medium (His+) or minimal medium lacking histidine (His-). In parallel, two independent transformants were assessed for *LacZ* activity each in triplicate, and values are expressed as Miller Units.
4.2. Mapping of the interaction domain.

The domains of CK2β that are responsible for mediating homotypic (β-β) or heterotypic (β-α) interactions have been established by a combination of biochemistry, two-hybrid analysis, and structural analysis on individual subunits and the holoenzyme. These studies demonstrate that a Cys4-zinc-finger motif in CK2β mediates homodimerization (Chantalat et al., 1999), whereas residues in the vicinity of the C-terminus mediate interaction with CK2α (Niefind et al., 2001) (Fig. 11A). We, therefore, aligned the C-terminal CK2α-interaction domain of all of the Drosophila β-like proteins, relative to the human protein, to clarify the level of conservation. While this domain is remarkably conserved amongst human β, fly β, and β', it is highly divergent in SSL and is virtually absent in Ste (Fig. 11B). We, therefore, generated deletions of the SSL protein to test whether this region also mediates interaction with CK2α. We find that, compared to full length SSL, truncation at Lys186 (K186*) decreases the affinity of interaction with CK2α by ~30%, whereas truncations at either Glu173 (E173*) or Gly148 (G148*) virtually abolish interaction (Fig. 11C). The residual reporter gene activity may represent a bridge interaction mediated via yeast CK2 subunits, which exhibit two hybrid interactions with their Drosophila homologs (Trott and Bidwai, unpublished). Control experiments where yeast expressed the truncation variants by themselves did not induce either reporter gene. These results demonstrate that, in spite of sequence divergence, the domain that mediates the SSL-CK2α interaction is "functionally" identical to that in β or β', and the equivalent affinities of these three proteins for interaction with CK2α (Fig. 10), further underscores the likelihood that these proteins can generate alternative holoenzyme isoforms in vivo.

4.3. Rescue of ion-homeostasis defects of ckb1 or ckb2 yeast:

We have previously observed that loss of either the β or β' subunit elicits defects in ion-homeostasis in S. cerevisiae (Bidwai et al., 1995), and this phenotype, elicited due to attenuated expression of the sodium-transport-pump (Tenney and Glover, 1998), is
Figure 11. Mapping of the SSL-CK2α interaction domain.

A. Schematic representation of the SSL protein illustrating motifs (not drawn to scale) conserved amongst all β-homologs; an N-terminal (auto)phosphorylation site, acidic loop, a Cys4-type zinc finger that mediates dimerization, and a domain (checkerboard) that mediates interaction with CK2α. B. Alignment of the CK2α-interaction domain in CK2β like proteins. White letters, identical residues; black letters, non-conserved substitutions; shaded letters, conservative substitutions. C. Constructs expressing SSL or the indicated truncations were transformed in yeast HF7C by themselves or in combination with CK2α. Two independent transformants, each in triplicate, were assessed for LacZ activity and values are expressed as Miller Units.
complemented by expression of DmCK2β. This system thus affords a bioassay for assessing the functions of all Drosophila β-like proteins, i.e., β', SSL, and Ste. Constructs that confer Gal-1/10 mediated expression of CK2β, β', SSL and Ste were generated as described (see Methods), and tested for suppression of the ion-homeostasis defect of YAPB7 (deletion of CKB1 encoding CK2β Bidwai et al., 1995) or JCR8 (deletion of CKB2 encoding CK2β' Reed et al., 1994). As expected, transformation of ckb1 or ckb2 strains with the expression vectors lacking an insert (pBM272 or pESC-URA) did not elicit any suppression (Fig. 12). On the other hand, transformation with a plasmid expressing CK2β elicited suppression, albeit incomplete, and comparable results were observed with cells expressing SSL. On the other hand, complementation with β' was at best marginal, while none was observed with the Ste protein. Taken together, the rank order of efficacy of these proteins would appear to be (CK2β~SSL)>β'.

We have previously demonstrated that Ste does not exhibit a two-hybrid interaction with CK2α, and the most likely reason is the absence of an α-interaction domain (Fig. 11B). These studies raise the obvious question why, inspite of comparable interaction with CK2α, β' exhibits the least functionality, and we believe that structural conservation of domains in metazoan β subunits provides a plausible explanation. Apart from residues mediating β-β or β-α interactions, all canonical β subunits conserve two "functional" domains; an N-terminal autophosphorylation site and an acidic domain (Fig. 12B). CK2β autophosphorylates at M^1SSPSEE and this, in turn, elicits phosphorylation of Ser^2 (M^1SSPSEE) because the consensus is S-D/E-x-D/E, and Ser^phos mimics Asp (Chantalat et al., 1999). Interestingly, this site in β' is M^1TDSDE and the presence of Asp^3 (instead of Ser^3 in β) raises the possibility that β' is 'constitutively phosphorylated', whereas the corresponding site in SSL (M^1SCPRS) is non-autophosphorylatable. It has been suggested that autophosphorylation stabilizes CK2β against ubiquitination and proteosomal degradation in mammalian cells (Zhang et al., 2002), but somewhat conflicting results have been obtained in Drosophila.
(Jauch et al., 2002), wherein non-autophosphorylating CK2β is equally competent at rescuing the lethality associated with a deletion of the CK2β gene. Our observation that CK2β and SSL, but not β', appear to be functional in the yeast system would suggest that autophosphorylation may not account for the functional differences. A more likely reason for the observed behavior of these β-like proteins is, perhaps, the acidic domain (Fig. 12B). Analysis of the structure of the holoenzyme (Niefind et al., 2001; Niefind et al., 1998), and references within) combined with kinetic analysis, suggests that this domain modulates substrate recognition and mediates activating effects of polybasic compounds such as spermine. In line with this, 7/10 residues of this region of CK2β, and 6/10 in SSL (both of which exhibit equivalent complementation, Fig. 12A) are either Asp/Glu, whereas that in β' is the least acidic (3/10, See Fig. 12B). If substrate recognition and/or targeting of CK2-activators is one of the functions of this domain, SSL would appear to be closest in function to CK2β and this is essentially reflected in the rank order of efficacy.

4.4. Real-time RT-PCR analysis of the expression of CK2β and SSL.

It has recently been suggested, based on Northern analysis, that β' and SSL are only expressed in adult Drosophila in a testis-specific manner (Kalmykova et al., 2002), a scenario that is at odds with our isolation of SSL cDNAs from embryo libraries. We have, therefore, used real-time RT-PCR, a more sensitive approach for detection of transcripts and one that also provides an assessment of relative transcript copy number. FAM-labeled probes specific to CK2β and SSL were designed as described in methods, and used to probe RNA isolated from the indicated developmental stages and sexes. We find that CK2β transcripts do not display sexual dimorphism (Fig. 13), whereas transcripts encoding SSL are easily detected in males at all of these stages, but appear undetectable in females. The essentially similar Ct values for CK2β in males versus females at all developmental stages tested would argue
Figure 12. Complementation of *ckb1* or *ckb2* yeast by Drosophila CK2β-like proteins.

A. Yeast strains lacking either *ckb1* (*ckb1-Δ1*) or *ckb2* (*ckb2-Δ1*) subunits were transformed with the expression vectors or vectors expressing CK2β, Ste, β', or SSL. Transformants were selected, and following growth in rich medium, dilutions were plated to rich galactose medium (YPG) or on YPG supplemented with 0.5M NaCl (YPGS). Plates were incubated at 29°C for 3-4 days and photographed. B. Alignment of the functional motifs in Drosophila CK2β-like proteins.
Figure 13. Real time RT-PCR analysis of CK2β and SSL.

Primers specific to CK2β and to SSL were designed as described in Materials and Methods, and used to screen RNA from single sex-selected third instar larva, pupa, and adult. Fluorescence output was captured and analyzed to calculate the threshold cycle (Ct). Higher Ct values correspond to a lower transcript copy number and Ct values greater than 32 represent levels that are non-detectable and are, therefore, indicated as N.D.
against the possibility that our inability to detect SSL transcripts in females is an artifact, and reflect the observation that levels/activity of CK2 appear constant throughout development and that CK2β is recessive lethal in males and females (Jauch et al., 2002). Our results clearly demonstrate that expression of SSL occurs across a wider developmental window than recently suggested (Kalmykova et al., 2002), and that SSL exhibits sexual dimorphism and thus is likely to be a male-specific gene. Our isolation of multiple cDNAs encoding SSL from an embryo-library would, at face value, also suggest expression at the earliest developmental time-point. However, attempts to probe RNA isolated from embryos via real-time RT-PCR suggest that transcript levels at this stage are below detectable levels (data not shown). We believe that our successful isolation of SSL transcripts from an embryo library are a reflection of the inordinately large number of primary two hybrid transformants (4x10^8), and that interaction of SSL with CK2α is the highest of any interacting partner in this two hybrid hunt. Our two hybrid screen may thus favor the isolation of relatively rare transcripts present at levels lower than those detectable via other means.

These studies raise obvious questions about the functions of SSL in a male specific manner. Although testicular development initiates early in development, meiosis does not initiate until adult eclosion. Our observation that SSL expression is also found in larval and pupal stages suggests that SSL's functions are not restricted to meiotic events, and may also be involved in the ontogeny of testis development. Our interaction data and deletion analysis demonstrate that like CK2β, SSL is capable of complexing with CK2α forming an alternative CK2 holoenzyme isoform, while differential abilities to rescue yeast would suggest functional differences. Future studies using β'/SSL transgenes for complementation of lethal/hypomorphic CK2β alleles and the global effort to isolate P-element insertions in all autosomal genes will provide insight into their functions.
Chapter 3.

1. Abstract

The Notch effector E(spl)M8 is phosphorylated at Ser\textsuperscript{159} by CK2, a highly conserved Ser/Thr protein kinase. We have used the Gal4-UAS system to assess the role of M8 phosphorylation during bristle and eye morphogenesis by employing a non-phosphorylatable variant (M8SA) or one predicted to mimic the 'constitutively' phosphorylated protein (M8SD). We find that phosphorylation of M8 does not appear to be critical during bristle morphogenesis. In contrast, only M8SD elicits a severe 'reduced eye' phenotype when it is expressed in the morphogenetic furrow of the eye disc. M8SD elicits neural hypoplasia in eye discs, elicits loss of phase-shifted Atonal-positive cells, i.e., the ‘founding’ R8 photoreceptors, and consequently leads to apoptosis. The ommatidial phenotype of M8SD is similar to that in \textit{N}\textsuperscript{\textit{spl}}/Y; \textit{E(spl)}\textsuperscript{D}/+ flies. \textit{E(spl)}\textsuperscript{D}, an allele of \textit{m8}, encodes a truncated protein known as M8\textsuperscript{*}, which, unlike wild type M8, displays exacerbated antagonism of Atonal via direct protein-protein interactions. In line with this, we find that the M8SD-Atonal interaction appears indistinguishable from that of M8\textsuperscript{*}-Atonal, whereas interaction of M8 or M8SA appears marginal, at best. These results raise the possibility that phosphorylation of M8 (at Ser\textsuperscript{159}) might be required for its ability to mediate ‘lateral inhibition’ within proneural clusters in the developing retina. This is the first identification of a dominant allele encoding a phosphorylation-site variant of an E(spl) protein. Our studies uncover a novel functional domain that is conserved amongst a subset of E(spl)/Hes repressors in Drosophila and mammals, and suggests a potential role for CK2 during retinal patterning.
2. Introduction

Throughout development, cell fate specification occurs with remarkable precision to generate a diverse array of cell types through the activities of highly conserved signaling pathways. One of these is the Notch pathway that is involved in the specification of a variety of cell fates (for reviews, see Artavanis-Tsakonas et al., 1995; Blaumuller and Artavanis-Tsakonas, 1997; Mumm and Kopan, 2000). This pathway consists of the receptor Notch (N), and other loci such as Delta (Dl), Suppressor of Hairless (Su(H)), and the Enhancer of Split complex (E(spl)). Among these, Dl encodes a ligand of Notch, Su(H) encodes a transcription factor, and E(spl) the ultimate transcriptional target of Notch signaling. The E(spl) locus encodes seven bHLH proteins (Mδ, Mγ, Mβ, M3, M5, M7, M8) and the non-bHLH protein Groucho (Gro) (de Celis et al., 1996; Delidakis and Artavanis-Tsakonas, 1991; Jennings et al., 1994; Klambt et al., 1989; Knust et al., 1992).

Much has been learned about the roles of Notch during neurogenesis in Drosophila. The first step during the process of neuronal development is the generation of a precise spatial pattern of specification of neural precursors. During this process, proneural bHLH proteins (henceforth referred to as bHLH-activators) encoded by the achaete-scute complex and atonal (ato), are expressed in groups of cells, the proneural clusters, within the anlagen of the CNS and PNS (Heitzler et al., 1996; Hinz et al., 1994), and this expression serves to maintain neural competency. However, all cells within proneural clusters do not adopt a neural fate even though they have the potential to do so. When one of these cells gains advantage over other cells of the proneural cluster, it goes on to adopt the neural fate and forces other cells to an alternative cell fate, such as epidermis. This altered cell fate specification has been termed 'lateral inhibition', and involves Notch mediated expression of E(spl) bHLH-repressors. Specifically, E(spl) bHLH proteins recruit Gro (a co-repressor) via an invariant C-terminal WRPW motif (reviewed in Chen and Courey, 2000; Fisher and Caudy, 1998) in order to antagonize the functions of bHLH-activators during bristle and eye morphogenesis (Giebel and Campos-ortega, 1997; Ligoxygakis et al., 1998). In line with this antagonism, loss of E(spl) elicits neural hyperplasia, while ectopic expression elicits neural hypoplasia; the latter effect is suppressed by concomitant overexpression of bHLH-activators such as L'sc (Giebel and Campos-ortega, 1997).
Among all of the cell fates regulated by Notch, its roles during eye development are, perhaps, the most complex (reviewed in Frankfort and Mardon, 2002; Hsiung and Moses, 2002). The Drosophila eye contains ~800 ommatidia (facets) that are arranged in a two dimensional lattice. Each facet is composed of eight photoreceptor neurons (R-cells) and 12 accessory cells that are arranged in a stereotypical pattern (reviewed in Freeman, 1997). One of the earliest steps in eye development is the process of retinal patterning during which the 'founding' R8 photoreceptors are specified. In this regard, patterning of the retina occurs in the wake of a wave of differentiation called the morphogenetic furrow (MF) that sweeps across the eye disc. During this process, the epithelium is systematically transformed into an hexagonal array of ommatidia, each of which is derived from a 'founding' R8 photoreceptor. Patterning of the eye thus reflects the arrangement of R8's that are established in the MF (White and Jarman, 2000). R8 specification requires the bHLH-activator Atonal (Jarman et al., 1995; White and Jarman, 2000), and involves signaling via EGFR, Notch, etc. (reviewed in Frankfort and Mardon, 2002; Hsiung and Moses, 2002; Kumar and Moses, 1997; Pichaud et al., 2001). In this context, Notch plays dual roles. In an early phase termed 'proneural enhancement', Notch elicits ato expression in 'intermediate' group cells (proneural clusters) (Baker et al., 1996; Baonza and Freeman, 2001). Subsequently, Notch mediates refinement (lateral inhibition) in the 'intermediate' group cells via E(spl) repressors, thus restricting ato expression to a single cell that goes on to differentiate as an R8 cell (Ligoxygakis et al., 1998). The remaining cells of the group do not adopt a neural fate, but remain uncommitted thereby ensuring their competency for subsequent recruitment (by the R8 cell) into the developing ommatidium.

It is, perhaps, unique to R8 specification that the dual functions of Notch (proneural enhancement and lateral inhibition) affect the same cell fate decision. Consistent with this, an absence of N during 'proneural enhancement' severely reduces ato expression and results in a loss of R8 cells (as seen in ato^ flies, Jarman et al., 1994; Ligoxygakis et al., 1998). In contrast, an absence of N during lateral inhibition prevents resolution of 'intermediate' groups into single phase-shifted R8 cells. In this case, as a consequence virtually all cells of the 'intermediate' group continue to express Ato and go on to adopt an R8 cell fate (Ligoxygakis et al., 1998). Predictably, E(spl) clones in the eye disc are compromised only in refinement and thus exhibit supernumerary R8 cells. Of the seven E(spl) genes, three (m8, mβ, and mδ)
are expressed in the eye disc (Cooper et al., 2000; Ligoxygakis et al., 1998). However, mδ is thought to play a predominant role in this tissue, because its overexpression and mutation in the $E(spl)^D$ allele (encoding M8*) severely attenuates retinal morphogenesis, and because the loss of $m\beta$ and $m\delta$ does not affect eye development (Nagel and Preiss, 1999; The et al., 1997). Lateral inhibition also plays a role in bristle morphogenesis where bHLH-activators, e.g., Ac, Sc, L'sc are antagonized by E(spl) repressors (Culi and Modolell, 1998; Giagtzoglou et al., 2003; Giebel and Campos-ortega, 1997; Nakao and Campos-Ortega, 1996).

Despite extensive studies on the roles of E(spl) proteins during bristle and eye development, it has remained unknown if their repressor activities are regulated in vivo, e.g., via phosphorylation. It has been known that ectopic expression of E(spl) repressors in wild type flies dominantly suppresses bristle development, but does not elicit eye (ommatidial) defects (Giebel and Campos-ortega, 1997). In addition, while E(spl)-repressors interact with bHLH-activators such as Ac, Sc, etc. (Alifragis et al., 1997; Gigliani et al., 1996), their interactions with Atonal are observed only upon deletion of the C-terminal domain, as with M8* (Nagel et al., 1999; Nagel and Preiss, 1999). While these discrepancies, perhaps, raised the possibility of different modes of function/regulation, the mechanism remained unknown. Our observation that a subset of E(spl) repressors, i.e., E(spl)M5, M7, and M8 are targeted by CK2, a Ser/Thr protein kinase, raised the possibility of phosphorylation as a regulatory mechanism (Trott et al., 2001b). CK2 is a highly conserved, messenger-independent, protein kinase with well defined roles in transcription, cell-cycle progression, checkpoint control, signal transduction, and development (reviewed in Bidwai, 2000; Glover, 1998; Meggio and Pinna, 2003). Some of its targets in Drosophila include Topoisomerase II, Antennapedia, Eve, Engrailed, Cactus, NAP1, HMG, Period, etc. (Akten et al., 2003; Bourbon et al., 1995; Corbett et al., 1992; Jaffe et al., 1997; Li and Manley, 1999; Li et al., 1999; Lin et al., 2002; Liu et al., 1997; Szewczuk et al., 1999). Consistent with multiple functions, many of which appear to be cell autonomous, loss of CK2 is lethal in yeast, mammals, and Drosophila (Buchou et al., 2003; Jauch et al., 2002; Lin et al., 2002; Padmanabha et al., 1990).

We have now analyzed the in vivo effect of the phosphorylation of E(spl)M8 by CK2 using variants that replace Ser$^{159}$ (the CK2 phosphoacceptor) with Ala/Asp. We find that a Ser$^{159}$-Asp variant, M8SD, dominantly interferes with eye development. We provide
evidence in favor of the proposal that CK2 might regulate the ability of M8 to antagonize Atonal in the eye. Our studies also suggest that the mechanism underlying the dominant ommatidial defect of M8SD is similar to that of $E(spl)^D$. Given that lateral inhibition by E(spl)M8 mediates refinement of the 'founding' R8 photoreceptors via antagonism of Atonal, our studies implicate a potential role for CK2 during eye development.
3. Materials and Methods

3.1. Plasmid Construction and Germ-line Transformation

The construction of variants of M8 harboring Ala/Asp in place of Ser159 (M8SA and M8SD) has been described previously (Trott et al., 2001b). For construction of N-terminal Flag-epitope (MDYKDDDDK) tagged M8, M8SA, and M8SD, the open-reading-frames were amplified by PCR using custom primers. All constructs were subcloned into the pBluescript-II (Stratagene, Inc.), and completely sequenced on both strands using the Prism Dye Terminator Cycle sequencing kit (Applied Biosystems). For in vivo expression, cDNAs were subcloned into the EcoRI and BamHI sites of the plasmid pUAST (Brand and Perrimon, 1993), and transgenic lines were generated by germ line transformations as described (Rubin, 1983). 

3.2. Flies and Phenotypic Analysis

Flies were raised at 25°C on standard Yeast-Glucose medium. The Gal4 lines used in these studies were either obtained from the Stock Center at Indiana University (Bloomington) or were kindly provided to us by Anette Preiss and Yuh Nung Jan. The Gal4 drivers used in these studies are, G455.2 and scaGal4 (Giebel and Campos-ortega, 1997), Gal4^{109-68} (Doherty et al., 1997; Frise et al., 1996), hGal4, gmrGal4 and sevGal4 (Ashburner, 1989). Balanced stocks of transgenic lines were generated according to standard procedures and crossed to Gal4 drivers. To minimize variability of the phenotype, crosses were performed at 25°C and employed 10-13 independent insertions of each transgene. For SEM analysis on the eyes, fly heads were passed thorough a graded alcohol series (25-50-75-absolute), and finally passed through Hexamethyldisalizane. Fly heads were sputter coated, and examined with a JEOL-6400 scanning electron microscope at an accelerating voltage of 20 kV. Images were collected and mounted with Adobe Photoshop and collated in Adobe Illustrator.
3.3. Immunocytochemistry and Westerns

Eye imaginal discs were isolated from late third instar crawling larvae, and processed as described (Kavler et al., 1999) with modifications. Eye discs were fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 15 min, and washed three times with PBS containing 0.1% Triton X-100 (PBS-TX). The discs were incubated for 12h at 4°C in PBS-TX containing 5% normal horse serum and mouse anti-Elav hybridoma supernatant (mAb 9f8A9) at a 1:100 dilution. The anti-Elav hybridoma supernatant developed by G. M. Rubin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained at The University of Iowa, Department of Biological Sciences, Iowa City, IA. Discs were washed three times with PBS-TX, and incubated with horse anti-mouse IgG-HRP conjugate (Vector laboratories) for 3 hours. Discs were stained by incubation in PBS-TX containing 0.5 mg/ml diaminobenzidine, 0.3% H$_2$O$_2$, and 1.5 mM NiCl, and reactions were stopped by extensive washing. Discs were dried, passed through a graded alcohol series (70-95-absolute), immersed in Hemo-D, and mounted in Cytoseal (Richard Allan Scientific). Slides were photographed and images mounted with Adobe Photoshop. Immunostaining of Ato was performed using a polyclonal rabbit serum (α-Ato, gift of Yuh Nung Jan) at a dilution of 1:5000 with 5% normal goat serum, followed by goat anti-rabbit IgG-HRP conjugate (Vector laboratories) at a dilution of 1:1000.

CK2 was purified from embryos using a procedure that will be described elsewhere. The purified enzyme displays a subunit composition, sedimentation coefficient, and kinetic parameters that are identical to those previously reported for the fly enzyme (Glover et al., 1983). A polyclonal rabbit antiserum against CK2 was a gift of Claiborne Glover and has been described earlier (Dahmus et al., 1984). Eye imaginal discs were isolated from crawling third instar larvae and solubilized in SDS-sample buffer. Extracts of 10 eye discs or purified CK2 (100 ng) were boiled for 10 minutes, clarified by centrifugation at 13,000xg for 5 min, electrophoresed on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose. CK2 subunits were detected using primary antibody against CK2 at a dilution of 1:1000, and secondary antibody (goat-anti-rabbit IgG coupled to alkaline phosphatase, Biorad) at a dilution of 1:3000. Immunoblots were visualized using nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (Ausubel et al., 1989). Immunostaining of CK2 was performed.
using a protocol similar to that described above for Ato, except that CK2 was detected using a polyclonal rabbit serum at a dilution of 1:100, and goat anti-rabbit IgG-HRP conjugate (Vector laboratories) at a dilution of 1:1000.

3.4. **Acridine orange staining**

Eye imaginal discs were isolated from late third instar crawling larvae in Drosophila Ringers, and stained with 1mM acridine orange (Sigma) for 5 minutes at room temperature. Discs were rinsed once in Ringers, and visualized on an Olympus AX70 fluorescence microscope. Images were collected using IP-Lab software.

3.5. **Two-hybrid analysis**

Interactions were studied in the LexA-based version of the interaction trap (Gyuris et al., 1993). Fusions with the activation domain (AD) employed either VP16 (Gro, CK2α) or B42 (Ato), and interactions were assessed in yeast EGY048. For quantitative assessment of interactions, three independent transformants, each in triplicate, were assayed for *LacZ* activity employing ONPG as a substrate as described (Trott et al., 2001b). *LacZ* activity was determined using the formula 1000xOD\textsubscript{420}/(TxVxOD\textsubscript{600}), where T is minutes and V is the concentration factor of the assay.
4. Results and Discussion

4.1. Structure of M8 and its CK2-specific variants

We previously reported that CK2 interacts with E(spl)M5, M7, and M8, but not with other E(spl) proteins (Trott et al., 2001b). This specificity of interaction correlated to the presence of a highly conserved CK2 site (Fig. 14A, inset). Consistent with this, CK2 phosphorylates these three bHLH proteins, and substitution of Ser\(^{159}\) of the M8 protein with Ala abolished phosphorylation. We focused on the analysis of M8 phosphorylation, because its roles during bristle and eye morphogenesis are well established (Giebel and Campos-ortega, 1997; Ligoxygakis et al., 1998; Nagel et al., 1999). For in vivo studies, we have employed two variants of M8 with respect to the CK2 site, S\(^{159}\)DCD (Fig. 14A). M8SA (Ser\(^{159}\)-Ala) is non-phosphorylatable whereas M8SD (Ser\(^{159}\)-Asp) is predicted to mimic the 'constitutively' phosphorylated protein. In addition, we have utilized M8*, a variant encoded by \(E(spl)^D\) that is truncated C-terminal to the Orange domain, and is thus lacking the CK2-phosphorylation and Gro-interaction sites (Klambt et al., 1989). Our decision to utilize Asp as a potential phosphomimetic residue is based on analyses of CK2 targets such as NAP1 and β-catenin, and protein kinases such as Aurora, where such substitutions 'mimic' the in vivo behavior of the phosphorylated protein (Li et al., 1999; Littlepage et al., 2002; Song et al., 2003). Like M8SA, M8SD is refractory to phosphorylation by CK2 (Trott et al., 2001b). This finding is critical since acidic residues are positive determinants for phosphorylation by CK2 (Meggio et al., 1994b), and confirms that replacement with Asp (M8SD) does not generate a second site for phosphorylation that may have confounded interpretations of phenotypes.

4.2. Characterization of M8 and its CK2-specific variants

Previous studies demonstrate that the C-terminal WRPW motif (Fig. 14A) mediates recruitment of the co-repressor Gro, and this interaction is required for proper in vivo function (repression) of all E(spl) proteins (reviewed in Chen and Courey, 2000; Fisher and Caudy, 1998). Thus it was critical to ascertain that CK2-specific variants of M8 interact with Gro, and for this purpose we have used the yeast interaction trap assay (Brent and Finley, 1997). In agreement with previous reports (Alifragis et al., 1997; Paroush et al., 1994),
interaction of Gro with M8 is robust, and similar results were obtained when Gro was tested for interaction with M8SA or M8SD (Fig. 14B). In contrast, the absence of WRPW in M8* prevents its interaction with Gro and has been described (Nagel et al., 1999), and Fig. 14B). Distinct differences were, however, observed when M8 variants were tested against CK2α (Fig. 14B). While CK2α interacts robustly with M8, its interaction with M8SA was attenuated by ~50%. In contrast, interaction of CK2α with M8SD appears marginal, at best, because LacZ values for this combination (~30 units) are close to the baseline (10-20 units) in this version of the yeast interaction trap. M8*, which lacks the CK2 site, did not interact with CK2α. These results raise the possibility that phosphorylated M8 has altered functions in vivo. Thus genetic analysis of M8, M8SA, and M8SD (see below) is likely to uncover the role of phosphorylation during neurogenesis, and the interpretations of phenotypes are unlikely to be confounded by simultaneously perturbed interactions with Gro and CK2α, as appears to be the case with M8*.

We have used the Gal4-UAS system (Brand and Perrimon, 1993) to explore the role of M8 phosphorylation. This approach has been widely used in Drosophila (for review, see Duffy, 2002), and has enabled analyses of the b/HLH, Orange, and WRPW domains of M8 during bristle and eye morphogenesis (Giebel and Campos-ortega, 1997; Ligoxygakis et al., 1998; Nagel et al., 1999). We have generated transgenic flies harboring UAS-m8, UAS-m8SA, and UAS-m8SD constructs, and multiple lines of each transgene were employed to eliminate position effects. The single UAS-m8* line was a gift from Anette Preiss and has been described previously (Nagel et al., 1999). We considered the possibility of employing a 'non-interacting' variant of M8 (M8ΔCK2) that lacks the CK2-site (SDCD). We have, however, deferred from in vivo analysis of this variant because, unlike M8SA or M8SD, it is weakly phosphorylated (by CK2) at cryptic site(s) (data not shown), perhaps, due to misfolding.

4.3. Role of M8 phosphorylation during bristle development

As stated above, E(spl) proteins inhibit neural fate by antagonism of bHLH-activators (Giatztoglou et al., 2003; Giebel and Campos-ortega, 1997; Nagel et al., 1999). In line with this, ectopic expression of E(spl) proteins elicits neural (bristle) hypoplasia, and such
Figure 14. Structure and interactions of M8 and variants.

A. Schematic of variants used in these studies. A and D represent Ala and Asp substitutions of Ser\textsuperscript{159}, of the CK2 consensus site (SDCD), and the deletion in M8* is as described (Giebel and Campos-ortega, 1997); (Nagel et al., 1999). B. LexA-fusions of M8 and variants were tested against Groucho- or CK2α-fusions to the activation domain of VP16. \textit{LacZ} activity is the average of 3 independent experiments each in triplicate. We have previously described the interaction of M8, M8SA, and M8SD (but not M8*) with CK2α (Trott et al., 2001a).
dominant effects should enable a dissection of the role of M8 phosphorylation during bristle morphogenesis.

To assess the ability of M8 variants to suppress bristle development, we initially elected to employ the Gal4 driver, G455.2, that restricts expression to the anlage of the scutellum (Giebel and Campos-ortega, 1997). Compared to 4 macrochaetes that are invariant on the scutellum of wild type flies (Fig. 15A), expression of M8 reduced the number of macrochaetes to 1±0.4 (± SD, Fig. 15B), while expression of M8SA or M8SD elicited a complete suppression (Fig. 15C and 15D). Similar phenotypes were observed with multiple insertions of each transgene. In contrast, expression of M8* resulted in 5.5±0.5 macrochaetes on the scutellum (Fig. 15E), indicative of an excess recruitment of sensory organ precursors (SOP). No such bristle abnormalities (hypoplasia or hyperplasia) were associated with the parental lines by themselves, or in progeny that harbored the Gal4 driver in combination with the CyO balancer chromosome (data not shown). The bristle hyperplasia of M8* is due to impaired lateral inhibition, because this phenotype is dampened by co-expression of wild type M8 and exacerbated in a background heterozygous for E(spl)C (Giebel and Campos-ortega, 1997). On the other hand, the similarity of bristle suppression with M8, M8SA, and M8SD, and that all three isoforms contain a functional WRPW motif (Fig. 15B), suggest that the phosphorylation state of M8 might not be critical for its repressor activity during bristle morphogenesis.

We were concerned that the restricted (scutellar) expression with G455.2 might dampen, and thus preclude a distinction of quantitative differences between M8, M8SA, and M8SD. We, therefore, utilized scaGal4 that drives expression in neural precursors in the embryo, in proneural clusters in the imaginal discs, and in the morphogenetic furrow (MF) of the eye disc (Hinz et al., 1994; Nakao and Campos-Ortega, 1996). Studies from the laboratory of Campos-Ortega (Giebel and Campos-ortega, 1997) have shown that expression of M8 by scaGal4 elicits a strong, but not complete, suppression of macrochaetes and microchaetes on the scutellum, thorax and head, whereas M8* elicits ectopic and/or duplicated macrochaetes indicating a defect in lateral inhibition. We reasoned that the greater expression domain of scaGal4 might enable us to distinguish between the repressor activities of M8SA and M8SD. As expected, expression of M8 by scaGal4 elicited potent
The effect on scutellar macrochaetes was assessed following expression of M8 and variants with the Gal4 driver, G455.2. Balanced stocks of UAS constructs were crossed to G455.2 as described, and bristle phenotypes of non-CyO progeny were assessed. In wild type (WT), the scutellum exhibits four macrochaetes that are positionally invariant. M8 expression elicits a partial suppression, M8SA and M8SD elicit complete suppression, while M8* elicits ectopic bristles (arrows).
bristle suppression, while M8* elicited ectopic bristles (data not shown). As seen with G455.2, expression of M8SD (13 independent insertions tested) by scaGal4 also elicited bristle suppression that was somewhat stronger than that observed upon expression of wild type M8 (data not shown). A similar analysis with M8SA was precluded because its expression by scaGal4 elicits embryonic lethality. This lethality was observed with all UAS-m8SA insertions (10 lines) either at 25°C or at 18°C (where Gal4 activity should have been lowered). Aside from this caveat, these results suggest that phosphorylation of M8 might weakly augment its antineurogenic properties, although we cannot rule out the possibility that this is simply due to enhanced expression or stability of M8SD. Thus, again the results indicate that phosphorylation of M8 is not overtly critical during bristle morphogenesis.

4.4. Role of M8 phosphorylation during eye development

An unexpected outcome of our studies using scaGal4 was a dominant 'reduced' eye phenotype associated with expression of M8SD, but not with wild type M8. To illustrate this phenotype, we have performed SEM's on eyes of wild type flies and those expressing M8 or M8SD. As expected, compared to flies expressing only Gal4 (Fig. 16A, D) or wild type flies (data not shown), overexpression of M8 suppressed ommatidial bristles but did not alter either facet morphology or number (Fig. 16B, E). While M8SD also suppressed ommatidial bristles, it elicited a severe attenuation of the number of ommatidia (Fig. 16C, F). A virtually identical phenotype is seen with all independent insertions of UAS-m8SD (13 lines), and the number of ommatidia in each case was reduced to ~25. No such defects were associated with progeny of these crosses that harbored scaGal4 and the CyO balancer chromosome (data not shown), suggesting that the ‘reduced eye’ correlates to the expression of M8SD. In addition, some of the ommatidia of scaGal4/+; UAS-m8SD/+ flies exhibited a dimpled, 'blueberry', phenotype (Fig. 16F, arrows). A similar 'reduced eye' (ommatidial) phenotype has also been described with Nspl/Y; E(spl)D/+ flies (Nagel et al., 1999), i.e., those expressing the truncated M8* protein (Fig. 14A and Fig. 16H, J). We, however, note that in our hands Nspl/Y; E(spl)D/+ flies typically exhibit 4-8 residual ommatidia (Fig. 16H, J), whereas those reported by Nagel et al. (Nagel et al., 1999) exhibit ~25, perhaps, reflecting differences in the genetic backgrounds, culture procedures, etc. In contrast, no reduced ommatidial phenotype was observed when scaGal4 was used to drive expression of UAS-m8* (Fig. 16G, I).
The effect on the eye was assessed following expression of M8 and variants with the driver, *scaGal4*. Balanced stocks of UAS constructs were crossed to *scaGal4*, and eye phenotypes of non-*CyO* progeny were assessed by SEM as described in Experimental Procedures. The genotypes are indicated. Magnifications are, A-C and G-H (200x), D-F and I-J (1000x). The arrows in panel F denote ommatidia exhibiting a 'blueberry' phenotype.
latter result is consistent with studies showing that ectopic expression of \textit{UAS-m8*} elicits a 'reduced' eye phenotype in an \textit{N}^{pl}/\textit{Y}, but not in a wild type (\textit{X}/\textit{Y}), background (Nagel et al., 1999). The relevance of \textit{N}^{pl} to the eye phenotype of \textit{M8* (E(spl)}^{D}) is discussed below.

Although it has been shown that a double dose of \textit{M8} (in the wild type) does not inhibit eye development (Giebel and Campos-ortega, 1997; Nagel et al., 1999), it is nevertheless possible that the 'reduced' eye of \textit{M8SD} (or for that matter \textit{M8*}) reflects a higher dosage of the protein, or is specific to these variants. However, a direct assessment of protein levels is precluded by the absence of \textit{M8}-specific antibodies and our observation that anti-Flag antibodies do not detect variants that harbor a single Flag-epitope at their N-terminus (data not shown). It is currently unknown whether this reflects N-terminal processing which might interfere with antibody-binding. Aside from this caveat, and to circumvent this potential problem, we have utilized between 10-13 independent insertions of each transgene (except for \textit{M8*}), and find that constructs harboring a Flag-tag elicit phenotypes identical to their untagged counterparts (data not shown).


As stated above, \textit{scaGal4} driven expression of \textit{UAS-m8SA} elicited embryonic lethality, thus precluding analysis of this variant in the eye. We hypothesized that if \textit{M8SD} represents a dominant allele in the eye (see Fig. 16C), then \textit{M8SA} by virtue of being refractory to phosphorylation by CK2 should more closely mimic the behavior of wild type \textit{M8} and not elicit a 'reduced' eye phenotype.

To circumvent the embryonic lethality of \textit{M8SA}, we have utilized the driver \textit{Gal4}^{109-68} (Frise et al., 1996). In a comparative analysis of \textit{scaGal4} and \textit{Gal4}^{109-68}, Doherty et al. (Doherty et al., 1997) indicate that expression with the latter driver is weaker. The 109-68 insertion drives Gal4 in subsets of proneural cluster cells, SOPs and their daughters, and has previously been used by White and Jarman (2000) to demonstrate that ectopic expression of a \textit{UAS-ato} construct triggers a rough eye phenotype due to excess recruitment of R8 cells in the MF. We reasoned that the attenuated expressivity of this driver might obviate the embryonic lethality of \textit{M8SA}, and thus permit a complete analysis of the CK2-specific variants of \textit{M8} in the eye. This is indeed the case. Compared to the driver \textit{Gal4}^{109-68} (Fig. 17A), expression of wild type \textit{M8} or \textit{M8SA} does not elicit a reduced eye phenotype (Fig.
Figure 17. Eye phenotypes of M8 and variants.

The effect on the eye was assessed following expression of M8 and variants with the driver, $Gal4^{109-68}$. Balanced stocks of UAS constructs were crossed to $Gal4^{109-68}$, and eye phenotypes of non-CyO progeny were assessed by SEM. The genotypes are indicated, and magnification is 200x. (E) Expression domains of Gal4-drivers relative to the morphogenetic furrow (MF) of the eye imaginal disc. Cells at stage 1 are at the anterior margin of the MF (solid line) and those at stage 4 are at its posterior margin as described (contd.)

<table>
<thead>
<tr>
<th>UAS-Lines</th>
<th>w1118</th>
<th>m8</th>
<th>m8SD</th>
<th>m8SA</th>
<th>m8k</th>
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<tbody>
<tr>
<td>scaGal4</td>
<td>WT</td>
<td>WT</td>
<td>~25</td>
<td>EL</td>
<td>WT</td>
</tr>
<tr>
<td>sevGal4</td>
<td>WT</td>
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<td>WT</td>
<td>WT</td>
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<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>
(Frankfort and Mardon, 2002). Arrow denotes MF progression. Cells expressing Ato are shown in black and those expressing E(spl)M8 in response to Notch signaling are shown in gray. Cell types in bold denote those showing strong expression with \textit{sevGal4}. (F) Summary of ommatidial phenotypes with eye disc specific expression of M8 and variants. WT denotes wild type, EL denotes embryonic lethal, the number of independent insertion lines is indicated in parenthesis, and the single \textit{UAS-m8*} line is as described (Nagel and Preiss, 1999).
17B, 4C), while expression of M8SD elicited a 'reduced' eye phenotype (Fig. 17D). We note that expression of M8SA does, however, lead to a slightly 'rough' eye phenotype that was not observed in either the Gal4 driver or upon expression of M8. Similarly, the reduced eye of M8SD also displays roughening which might be compounded by the reduced eye field. The basis of the rough eye in M8SA expressing flies is currently under investigation. When the results with Gal4<sup>109-68</sup> are compared to those with scaGal4, it is evident that the M8SD reduced eye is more severe with the latter driver (compare Fig. 16C with Fig. 17D). This attenuated effect of M8SD is consistent with the reported differences between the two drivers; i.e., Gal4<sup>109-68</sup> being the weaker. Once again, no such ommatidial defects were associated with progeny that harbored the Gal4 driver and the CyO balancer chromosome (data not shown), suggesting that the 'reduced' eye phenotype is specific to M8SD.

Given the dynamics of MF progression and the mechanism of retinal patterning (see Introduction), we wanted to determine if the dominant eye phenotype of M8SD was specific to expression in the MF, the zone where R8 specification and refinement occurs. For these studies, we have used the drivers hGal4, gmrGal4, and sevGal4. Expression with hGal4 is anterior to the MF (the proliferative zone of the eye disc), that with gmrGal4 is in all cells posterior to the MF, and with sevGal4 expression is during secondary recruitment of cells into the assembling ommatidia (Fig. 17E). However, expression of M8SD did not elicit any reduced eye phenotype with these three drivers (Fig. 17F). These results suggest that the ommatidial defects of M8SD correlate to expression (via scaGal4 or Gal4<sup>109-68</sup>) in the MF, where lateral inhibition by E(spl)M8 mediates refinement of the 'founding' R8 cells via antagonism of Ato (Ellis et al., 1994; Lee et al., 1996; Ligoxygakis et al., 1998; Powell et al., 2001). The inability of M8SD to elicit a 'reduced' eye phenotype when expressed with gmrGal4 or sevGal4 either reflects differences in the strengths of these drivers or suggests that once R8 specification has been achieved, subsequent cell fate/functions become refractory to antagonism by M8SD. The latter interpretation would be consistent with the proposal of Nicholas Baker and coworkers that E(spl)M8 functions in the eye are dispensable following R8 differentiation (Ligoxygakis et al., 1998). The inability of M8* to elicit a reduced eye with hGal4 or scaGal4 is due to the fact that these crosses were conducted in wild type (X/Y), instead of an N<sup>pl</sup> background, and has been described by others (Nagel et al., 1999; Nagel and Preiss, 1999, and see below). Together, our results with scaGal4 and...
Gal4 further support the notion that M8SD is likely to represent a dominant allele of m8 in the eye.

4.6. Immunostaining with mAb-Elav, α-Ato, and α-CK2

To assess retinal patterning at an earlier developmental time-point, we have analyzed eye discs for the neuronal marker, Elav (Campos et al., 1987). This analysis is based on the observations that E(spl) clones in the eye disc exhibit enhanced neurogenesis in 'intermediate' group cells (Ligoxygakis et al., 1998). Additionally, the partial loss of ato disrupts lattice arrangement of R8's posterior to the MF, whereas its misexpression elicits supernumerary R8's in eye discs and rough eyes (White and Jarman, 2000). For these studies, we elected to utilize scaGal4 to drive UAS-m8 or UAS-m8SD because this driver provided the most severe phenotype. We find that Elav staining is dramatically attenuated in discs expressing M8SD (Fig. 18C), whereas those expressing M8 (Fig. 18B) appear to be similar to the wild type (Fig. 18A). The number of Elav-positive clusters in discs expressing M8SD roughly approximates the ommatidial number in the residual eye (Fig. 16C), and these appear to be randomly dispersed throughout the disc epithelium behind the MF (the site of expression) and posterior to it (Fig. 18C). A higher magnification of the region of staining in M8SD discs (Fig. 18C, inset) indicates that some of these ommatidia appear to contain a normal complement of photoreceptors while others appear aberrant. Thus the eye phenotype of M8SD reflects severe neural hypoplasia during ommatidial development.

To further refine the mechanism for the severe neural hypoplasia in M8SD eye discs, we have also assessed expression of Ato, a bHLH-activator required for R8 specification. As stated above, Ato expression within the MF is broad and ubiquitous at its anterior margin, whereas it resolves (due to antagonism by E(spl)M8) into a series of phase-shifted Ato-positive cells, the 'R8 founders' at its posterior margin. In addition, Nspl/Y; E(spl)D/+ eye discs exhibit a virtual ablation of these phase-shifted R8 founders, suggesting that the truncated M8* protein dominantly exacerbates lateral inhibition (refinement) of 'intermediate' group cells (Nagel and Preiss, 1999 and Fig. 18G). The similarity of eye defects raised the possibility of a similar mechanism for M8SD. We have, therefore, utilized an antibody (α-Ato) to visualize Ato levels in scaGal4 eye discs (Fig. 18D), and in discs wherein scaGal4 was used to express either UAS-m8 or UAS-m8SD (Fig. 18E, F). Consistent with the normal
ommatidial phenotype of $scaGal4/+; UAS-m8/+$ flies (Fig. 16B, E), expression of M8 does not appear to affect refinement of R8 'founders' which are visible in the micrograph as a series of phase-shifted Ato-positive cells (Fig. 18E). In contrast, expression of M8SD (with $scaGal4$) severely reduces the number of these phase-shifted R8 cells (Fig. 18F), although it appears to reduce Ato-positive cells at the anterior margins of the MF as well. The virtual loss of phase-shifted R8's upon expression of M8SD at this developmental time-point appears remarkably similar to that in $N^{spl}/Y; E(spl)^D/+\text{ discs}$ (Fig. 18G). Taken together, these results argue in favor of the possibility that the M8SD eye phenotype (like that of $E(spl)^D$) reflects exacerbated 'refinement' of R8 'founders'. Taking into account the outcome of Elav- and Ato-immunostainings, it is likely that a few R8 'founders' escape this developmental block. However, some of these might still be compromised for recruitment of secondary photoreceptors, thus resulting in an aberrant complement of Elav-positive cells in M8SD discs (Fig. 18C).

Given the potential role for CK2 in regulation of M8 in the eye, and despite its cell autonomous functions, we have assayed for its presence in the eye disc. Using an antiserum that recognizes both ($\alpha$ and $\beta$) subunits of Drosophila CK2 (Dahmus et al., 1984), we have conducted Western using either purified CK2 or extracts from eye discs. We find that CK2 is, in fact, present in eye discs (Fig. 18, $\alpha$-CK2, lane-Disc). Based upon parallel analysis with purified CK2 (Fig. 18, $\alpha$-CK2, lane-C), the mobility of the two bands in eye disc extracts corresponds to that of CK2$\alpha$ and CK2$\beta$. The differences in the intensity of staining of CK2$\alpha$ vs CK2$\beta$ in eye discs reflects a differential interaction of this antiserum with CK2 subunits and has been previously described by us (Bidwai et al., 1992). We have also used this antiserum to immunostain eye discs, and find uniform staining of all cells (data not shown). Our observation that CK2 is widespread and not restricted just to cells in the MF, appears consistent with its pleiotropic functions (see Introduction).

4.7. Exacerbated apoptosis in eye discs upon expression of M8SD

It has been shown that eye discs from $ato^{1}/Df(3R)p^{13}$ larvae (lacking Ato protein) exhibit a complete absence of R8 ‘founders’ and this manifests as pervasive apoptosis behind the MF (Jarman et al., 1995). This apoptosis is thought to reflect an absence of inductive cues that would normally be provided by R8 photoreceptors during the process of
Figure 18. Staining of eye discs.

Eye discs of the indicated genotypes were isolated and processed as described in Experimental Procedures. (A-C) mAb-Elav, (D-G) α-Atonal, and (H-J) acridine orange. White arrow in panels A-C and H-J denotes the MF, the bracket in panels D-G indicate the position of phase-shifted R8 founders. Only the relevant area of the eye disc is shown for α-Ato immunostaining. Western on eye disc extracts was performed using an antibody to CK2 (α-CK2). This antibody recognizes the α and β subunits of Drosophila CK2 (Gel). CK2 was detected in extracts of eye disc (lane-Disc) or on 100 ng of purified CK2 as a control (lane-C).
recruitment of other R-cells and accessory cells into the ommatidia (Jarman et al., 1995). If this is the case, the severe loss of R8 'founders' upon expression of M8SD might also manifest as enhanced apoptosis. Using acridine orange to label cell death, we find that discs derived from *scaGal4/+; UAS-m8SD/+* larvae do, in fact, display pervasive and biphasic apoptosis behind the MF (Fig. 18J). In contrast, discs derived from either wild type larvae (data not shown), *scaGal4* (Fig. 18H) or *scaGal4/+; UAS-m8/+* larvae (Fig. 18I) exhibit low levels of apoptosis that is characteristic of this tissue during this developmental time-point and has been described by others (Wolff and Ready, 1991).

### 4.8. Interaction with Atonal

The *E(spl)D* eye phenotype is due to an exacerbated physical interaction of the truncated M8* protein with Ato, whereas full length M8 either does not interact, or at best interacts very weakly (Nagel and Preiss, 1999). Given the similarity of attenuated R8 ‘founders’ upon expression of M8SD, we reasoned that this variant might also display exacerbated interaction with Ato, and, if so, would further strengthen the notion that M8* and M8SD employ common mechanisms. We have, therefore, tested for this interaction via the yeast interaction trap. We find that the M8SD-Ato interaction appeared virtually identical to that between M8*-Ato, whereas interaction of wild type M8 with Ato was negligible (Fig. 19A). In our hands, ≤ 10 Miller units represents values typically obtained for non-interacting proteins, e.g., M8*-Gro or M8*-CK2α (see Fig. 14B). Thus the *LacZ* values obtained for M8-Ato (11 Miller units) probably reflect the absence of an interaction. This interpretation is supported by the observation that the M8SA-Ato interaction gave results (9 Miller units) indistinguishable from those with M8-Ato. That *LacZ* values for M8SD-Ato interaction (~45 Miller Units, Fig. 19A) are significantly lower than those for M8SD-Gro (~600 Miller Units, Fig. 14B), does not imply a lower interaction affinity for the former protein pair. Rather, we attribute this difference to the weak activator from protein B42 that was used for the Ato analysis, rather than the strong activator from VP16 that was used for the Gro studies. That the interactions of M8* and M8SD with Ato are indistinguishable, suggests that the exacerbated interaction of M8SD with Ato is likely to also account for its ‘reduced’ eye phenotype. We note that expression with *scaGal4* and *Gal4*~109-68~ coincides with cells at stages 2/3 (Fig. 17E), a region of the MF where clusters of Ato-positive cells
undergo refinement via E(spl)M8. Our results with mAb-Elav and α-Ato, and direct interactions with Ato, suggests that M8SD exacerbates R8 refinement.

What then is the relevance of Nspl? We reiterate that E(spl)D (M8*) elicits opposite effects, i.e., bristle-hyperplasia that is Nspl-independent and photoreceptor-hypoplasia that is Nspl-dependent. This is not the case with M8SD which elicits bristle and photoreceptor loss in wild type (X/Y) flies. It has been shown that the broad and ubiquitous MF-specific expression of Ato in stage 1 cells (Fig. 17E) is attenuated in Nspl/Y discs, suggesting that this allele is compromised for 'proneural' enhancement (Nagel and Preiss, 1999). On the other hand, no such defects in Ato expression are associated with E(spl)D/+; X/Y discs (Nagel and Preiss, 1999). It is only in the Nspl/Y; E(spl)D/+ combination that eye defects become apparent. The truncation in M8* is likely to engender structural perturbations, and its inability to interact with the co-repressor Gro might well make it a weak repressor. As a result, M8* requires a 'sensitized' Nspl background where Ato levels are depressed. In contrast, M8SD harbors a single replacement (Ser159→Asp), and its competency to interact with the co-repressor Gro and with bHLH-activators such as Ato (Figs. 14B and 19A) as well as Sc and Ac (data not shown), suggest that it might not be structurally compromised. These features probably obviate the requirement of M8SD for a 'sensitized' Nspl background, and thus account for its consistent dominant behavior during bristle and eye morphogenesis.

4.9. Conservation of a CK2 consensus motif

Although E(spl) bHLH proteins are moderately conserved, they do display regions of similarity such as the b/HLH and Orange domains while the WRPW motif is invariant (Delidakis and Artavanis-Tsakonas, 1991; Maier et al., 1993). In contrast, residues between the Orange domain and WRPW are not highly conserved, and this region (of unknown function) accounts for a majority of the length heterogeneity. One notable exception, however, is a conserved 'subdomain' in the M8/M7/M5 proteins that contains an invariant CK2 consensus site (Fig. 19B). A strikingly similar sequence is also present in the human/murine bHLH repressor Hes6, and in a manner similar to that of the E(spl)M8-Ato interaction, phosphorylation of the CK2 site in Hes6 promotes its interaction with the proneural bHLH-activator Hes1 (Gratton et al., 2003). The high conservation of this region and its CK2 site in E(spl)M8, and our observation that perturbation of the CK2-
Figure 19. Interaction with Atonal and conservation of phosphorylation domain.

A. Interaction of M8 variants with Atonal. LexA-fusions of M8 and variants were tested against a B42-Ato fusion, and LacZ activity is the average of 3 independent experiments each in triplicate. B. Conservation of the phosphorylation domain in E(spl)/Hes proteins. Dh denotes Drosophila hydei, and hHes6 and mHes6 denote the human and murine Hes6, respectively. The CK2 site is marked by a bracket, asterisk marks the CK2 phosphoacceptor (shown in green), and other potential phosphoacceptors in the vicinity of the CK2 site are shown in red.
phosphoacceptor significantly alters its in vivo function(s), makes a plausible case for an evolutionarily conserved role for phosphorylation. We note that this phosphorylation domain (P-domain) also conserves a number of Ser and Tyr residues, as well as several hydrophobic and acidic residues. This raises the question of whether differential phosphorylation of the P-domain serves to 'fine-tune' M8 repressor activity, or simply acts as a modular phosphorylation-dependent switch. Such a modulation of M8 repressor activity that appears to be precipitated by a potentially phosphomimetic replacement also invokes questions on the roles of phosphatases that might oppose CK2. A reversal of M8 repressor activity by dephosphorylation might be relevant during retinal morphogenesis, since it could avoid a protracted block of the neural cell fate, and thus permit their secondary recruitment (as other R-cells, cone cells, etc.) into the assembling ommatidia.

4.10. Role for CK2 in Notch mediated lateral inhibition

How might phosphorylation of E(spl)M8 confer an ability to antagonize Ato? Our observation that the M8SD-Ato interaction is indistinguishable from that of M8*-Ato, suggests that, in an unphosphorylated state, the P-domain masks ('autoinhibits') the Orange domain (the site for Ato-binding) directly or indirectly (Fig. 20A). In this scenario, phosphorylation of Ser\(^{159}\) by CK2 would displace the blocking residues and permit binding to Ato and antagonism of its proneural functions. The dominant phenotypic effects of M8* (E(spl)\(D^D\)) described by Nagel and Preiss (1999) might be explained by the absence of such a putative 'autoinhibitory' domain. As stated above, a similar phosphorylation type switch is now also thought to mediate interaction of human/murine Hes6 with Hes1 (Gratton et al., 2003), although in their studies truncations of Hes6 (akin to M8*) were not tested.

Our studies, in combination with those on E(spl)\(D^D\) (Nagel et al., 1999), also suggest a potential mechanism for the role of CK2 in mediating Notch functions during retinal patterning (Fig. 20B). We note that this model is limited by the absence of eye-specific alleles of CK2, and that it is based on misexpression phenotypes of M8SD in the eye. Nevertheless, it takes into account the similar eye phenotypes of M8SD and E(spl)\(D^D\), and the known role of E(spl)M8 in mediating R8-refinement via antagonism of Ato. In this context, overexpression of M8, by itself, is not sufficient to allow for antagonism of Ato, an interaction that appears to require prior modification of the M8 protein via phosphorylation.
Figure 20. Proposed model for the role of CK2 function of M8

A. Mechanism for CK2 modulation of the M8-Ato interaction. Gray box, CK2 interaction/phosphorylation site; black box, Ato binding site; dotted line, blocking of the Ato binding site via non-covalent interactions; and PSer\textsuperscript{159} is the CK2 modified residue. B. Model for role of CK2 during photoreceptor refinement. Solid arrow, Ato-positive feedback loop; PPase, protein phosphatase; and R8, 'founding' R8 photoreceptor.
In this model, CK2 phosphorylates M8 in cells undergoing Notch-mediated lateral inhibition (stage 2/3 in the MF, see Fig. 17E), thus permitting antagonism of Ato and thereby the refinement of the R8 cells. The phenotypes of M8SD and M8*, albeit in different genetic backgrounds, and their interactions with Ato would appear to be consistent with this model. That M8SD perhaps mimics the 'constitutively' phosphorylated form of M8 might well underlie its dominant effects. In contrast, the absence of eye defects upon expression of wild type M8 probably reflects proper regulation via reversible phosphorylation in a precise spatial/temporal context, as would be achieved in wild type flies.

To our knowledge, this is the first suggestion that CK2 might regulate M8 repressor activity during retinal morphogenesis. If so, it would suggest an additional layer of complexity to Notch signaling than previously recognized. Future efforts to elucidate the structural alterations in M8 that are triggered upon phosphorylation by CK2, and the identification of any additional protein kinases and/or phosphatase(s) will help unravel the diversity of mechanisms regulating M8 during eye morphogenesis.
Chapter 4.

Analysis of the eye phenotypes due to CK2 specific variants of M8.
1. Abstract

The repressor activity of E(spl)M8 is regulated during neurogenesis via CK2 phosphorylation. Overexpression analysis of M8 and its CK2 specific variants resulted in three distinct outcomes. First, the expression of wild type M8 did not elicit any eye defects (reduced/rough). Second, M8SA elicits a rough (but not reduced) eye. Third, M8SD elicits to a severely reduced eye due to a dominant exacerbated antagonism of the proneural protein Atonal (Ato). The studies described in this chapter better define the molecular mechanism(s) underlying these developmental outcomes. We demonstrate that the reduced eye due to ectopic M8SD is completely suppressed by the simultaneous expression of Ato, indicating that Ato is the primary target of this dominant allele. In addition, a decrease in the dosage of CK2 partially attenuates the eye phenotype of M8SD, suggesting that endogenous M8 (phosphorylated by CK2) contributes to the eye phenotype. Furthermore, we demonstrate that the rough eye of M8SA reflects the presence of supernumerary R8’s, raising the possibility that this variant might function as an antimorph. This possibility has been confirmed. The effects of M8SA are, in fact, further exacerbated by the coexpression of Ato. Thus the antimorphic behavior of this protein reflects its interference with the normal functions of endogenous M8. Finally, we have analyzed the role of CK2 phosphorylation domain (CTD). We find that the expression of the isolated CTD weakly interferes with the functions of endogenous M8. The implications of these studies are discussed.
2. Introduction

During eye morphogenesis, the eye imaginal disc epithelium is systematically transformed into a hexagonal array of ommatidia, each of which is derived from a ‘founding’ R8 photoreceptor (Frankfort and Mardon, 2002; Hsiung and Moses, 2002). The specification of the R8 photoreceptor neuron occurs in the morphogenetic furrow (MF) and is dependent on the proneural bHLH transcriptional activator Atonal (Ato), (Jarman et al., 1995; White and Jarman, 2000). As mentioned previously and in this context, Notch signaling, in a biphasic manner, mediates the (spatially) precise specification of R8 cells. In the first phase (anterior to the MF), Notch elicits a broad expression of Ato, which serves to maintain (pro)neural competency (Baker et al., 1996; Baonza and Freeman, 2001). In the second phase, the expression of Ato resolves to individual cells that are then destined to become the future R8 photoreceptors (Ligoxygakis et al., 1998). This resolution is mediated through the induction of the E(spl) bHLH repressors by Notch. Amongst the E(spl) bHLH repressors, E(spl)m8 is thought to play a predominant role in the precise patterning of R8 cells via its ability to antagonize Ato. In this context, it is important to note that a variety of protein-protein interactions underlie the functions of M8. Structurally, the M8 protein harbors the N-terminal b/HLH domain, an internal HLH domain (Orange), a C-terminal domain that mediates interaction with and phosphorylation by CK2, and the penultimate tetrapeptide, WRPW, that recruits the co-repressor Gro. While the first HLH domain mediates homo/hetero dimerization amongst E(spl) members, the Orange domain mediates its interactions with Ato. As described in chapter 3, we have analyzed the role of phosphorylation of M8 via the targeted misexpression of two CK2 phosphorylation site-specific variants, M8SA and M8SD. The rationale underlying these variants has been described in chapter 3 and thus will not be reiterated here. The analyses of these variants implicate a role for CK2 in the regulation of the repressor activity of M8. Briefly, ectopic M8 elicits loss of Inter Ommatidial Bristles (IOB’s) but does not affect photoreceptor (PR, ommatidial) development. In contrast, ectopic M8SD elicits a severe arrest of IOB and PR development. Surprisingly, however, ectopic M8SA did not mimic M8, and resulted in a rough, but not reduced, eye. Cell fate mapping and protein-protein interaction analyses suggest that the M8SD phenotype reflects a dominant block of Ato, and mimics the effects of
M8*, a truncated variant of M8. M8* lacks sequences after the Orange domain, specifically the (C-terminal) CK2 and Gro interaction sites (Nagel and Preiss, 1999). We refer to this region as the M8CTD. Because of the phenotypic similarity of M8SD and M8*, we reasoned that the CTD autoregulates M8 repressor activity. Specifically, in M8 the CTD blocks the functionality of the Orange domain, thus precluding its interaction with Ato, and this block (autoinhibition) is overcome by phosphorylation. A model describing this autoinhibition has been discussed in chapter 3.

Based on the aforementioned studies, we propose the following hypotheses, each of which has been addressed via biochemical, molecular and genetic approaches. These are outlined below.

1. We have tested the hypothesis that exacerbated antagonism of Ato by M8SD is the primary reason for the reduced eye phenotype of this M8 variant.

2. We have tested the hypothesis that the effects of M8SD can be dampened by a simultaneous reduction in the dosage of CK2.

3. We have tested the hypothesis that the rough eye phenotype of M8SA is due to its interference with endogenous M8, i.e., M8SA is an antimorph.

4. We have tested the hypothesis that the CTD serves to autoinhibit the M8 protein during eye development.
3. Methods

3.1. Construction and Germ-line Transformation of CTD variants

CTD of M8 and of the variants harboring Ala/Asp in place of Ser159 (M8SA and M8SD) were generated by PCR and sequenced. The CTD’s were subcloned into different plasmids for various analyses such as yeast two hybrid analysis, recombinant expression, and *in vivo* analysis (Brand et al., 1994). Transgenic animals were generated by germline transformation and the location of insertions was determined via crosses to lines harboring chromosomes carrying dominant visible markers.

3.2. Flies and Phenotypic Analysis

Stocks harboring the full-length (UAS) transgenes M8/M8SA/M8SD and the Gal4 driver’s (*scaGal4* (Giebel and Campos-ortega, 1997), and *Gal4*109-68) are described in chapter 3. The CK2 mutant stock (*Tik*/Tm3, Sb) was a gift from Ravi Allada, Northwestern University. To minimize variability of the phenotype the flies were raised at 25°C on standard Yeast-Glucose medium, and employed at least 3-5 independent insertions of each transgene. For SEM analysis on the eyes, fly heads were processed as described in the previous chapter.

3.3. Immunocytochemistry

Immunostainings of eye imaginal discs were performed using the methods described in chapter 3. The following antibodies were used in this study: mouse anti-Elav at 1:100 (from DSHB, Iowa), guinea pig anti-senseless antibodies 1:500 (a gift from the laboratory of Hugo Bellen, HHMI, Baylor College of Medicine), Donkey anti-mouse Alexa flour 488 (1:500) and donkey anti-guinea pig Alexa flour 546 (1:500, from Molecular Probes). The eye discs were mounted in Vectashield and images were acquired with an Olympus XL confocal microscope and processed using Adobe Photoshop and Adobe Illustrator.

3.4. Two-hybrid analysis and kinase assays

Interactions were studied in the LexA-based version of the interaction trap. Interactions between the fusions with the activation domain (Gro, CK2α) and those with LexA DNA binding domain (CTD’s) were assessed as described in chapter 3.
Purification and phosphorylation of M8CT, SACT and SDCT was carried out as described by Trott et al., 2001.
4. Results and Discussion:

4.1. The reduced eye phenotype due to M8SD can be rescued by overexpression of Atonal

As stated above, M8SD antagonizes Ato thus dominantly blocking formation of R8’s, thereby leading to a reduced eye phenotype. If Ato represents the only target of M8SD, it would be reasonable to expect that the simultaneous overexpression of Ato should attenuate the dominant effects of M8SD. We note that the dominant reduction of the eye field due to ectopic M8SD reflects the strength of the Gal4 driver. For example, as described in chapter 3, *scaGal4* leads to a drastic reduction of the eye (5-15 ommatidia), while the 109-68Gal4 driver causes a less severe effect (~300 ommatidia). We, therefore, reasoned that the recapitulation of eye development might be more amenable with the weaker driver, 109-68Gal4. For these studies, UAS-m8SD and/or UAS-ato were overexpressed with 109-68Gal4 and eye phenotypes were analyzed by SEM. As a control, the simultaneous overexpression of Ato was carried out in flies expressing wild type M8. We find that the simultaneous overexpression of Atonal reversed the effects of M8SD (compare Fig. 21C with 21D). A close examination of Fig. 21D reveals that the severely disrupted hexagonal patterning is also restored. This patterning appears highly similar to that seen in wild type flies or in flies expressing M8 alone (Fig. 21A), or those coexpressing Ato+M8 (Fig. 21B). This rescue of the M8SD effect by ectopic Ato provides strong evidence for their interaction in vivo. These results support the hypothesis that M8SD defects are principally mediated through Atonal, and do not represent any other molecular targets of this dominant repressor.

4.2. Effects of reduced dosage of CK2 on the reduced eye defect of M8SD

The transgenic analyses in chapter 3 suggests that phosphorylation by CK2 is an essential step for the repressor activities of M8 during eye morphogenesis. Therefore, it is possible that the reduced eye phenotype due to ectopic expression of M8SD is an additive effect of the transgene-derived M8SD protein and the endogenous M8, in its
Figure 21. Simultaneous expression of M8 and M8SD with Atonal under Gal4109-68

Rescue of the M8SD eye phenotype by Ato overexpression. Effect of a simultaneous overexpression of Ato on the eye phenotypes due to expression of M8 and M8SD with the driver $Gal4^{109-68}$. The genotypes are as indicated. The eye phenotype as analyzed by light micrographs.
Figure 22. Expression of M8SD in flies harboring Tik mutation

Rescue of the M8SD reduced eye phenotype due to lowered dosage of CK2. Compare the eye phenotype of M8SD under the driver scaGal4 A with expression of M8SD in flies harboring the Tik mutation B. Tik encodes for a catalytically dead subunit (200X). C shows the blueberry phenotype of the ommatidia (1000X).
phosphorylated form. If this is indeed the case, a reduction in CK2 activity should partially restore the eye field by removing/attenuating the contribution of endogenous M8 protein. To test this hypothesis, we overexpressed M8SD in flies heterozygous for the mutant CK2α allele Tik, which encodes for a catalytically dead subunit (Lin et al., 2002). We find that overexpression of M8SD in a Tik background does not affect the reduced eye phenotype (compare Fig. 22A, B). These results suggest that the expression of M8SD isoform might be able to compensate for the decreased amount of phosphorylated endogenous M8. On the other hand and given that this analysis was conducted in a background heterozygous for Tik, it is possible that the decrease in the level of CK2 is insufficient to engender a noticeable change in the phosphorylation status of endogenous M8.

A higher magnification of the reduced eye phenotypes via SEM reveals the presence of uniformly dimpled ommatidia in the Tik/+ background. This was not the case when studies of ectopic M8SD were conducted in a background wild type for CK2 (compare Fig. 22C, D). These ‘dimpled’ ommatidia are suggestive of a defect during specification of one of the accessory cell fates, for example, cone cells (Basler et al., 1990). Cone cells are specified from a group of cells called the ‘R7 equivalence group’, and secrete the lens that gives the typical convex morphology to each ommatidium. The role of cone cell specification from the ‘R7 equivalence group’ is mediated by Notch signaling, but the roles of CK2 and/or E(spl) in this context are unknown. Future studies aimed at cell-fate analysis will be required to address the mechanism underlying the dimpled phenotype we have observed. One example of such an analysis would be to analyze the R7 specific markers Rh3/4, and the cone cell specific markers Cut and dPax2 in eye discs derived from scaGal4/UAS-m8SD; Tik/+.

4.3. Analysis of the rough eye phenotype of M8SA

During the analysis of the role of phosphorylation of M8 by CK2, we uncovered that the expression of M8SA led to a rough eye phenotype (see chapter 3, Fig. 17C). This rough eye reflects altered phasing of the ommatidia, which, unlike M8SD, was not accompanied by a reduction in the eye field. In addition this roughening was not observed in flies overexpressing M8 indicating that these defects are not due to simple overexpression, but are
specific to the substitution of the CK2 phosphoacceptor in M8.

To assess the effects of ectopic M8SA at an earlier stage of eye development, i.e.,
during R8 photoreceptor specification, eye discs were analyzed for the expression of the R8
specific marker Senseless (Sens) (Nolo et al.), and Elav (Campos et al., 1987), a terminal
neuronal marker. The staining of eye discs from larvae that overexpress M8 represents the
wild type pattern for both of these markers (Fig. 23A, B). Consistent with the rough eye
phenotype, the eye discs from larvae expressing M8SA exhibit the presence of
supernumerary R8’s, which results in a loss in their phasing as compared to discs expressing
wild type M8 (compare Fig. 23A with 23C). A simultaneous staining of M8SA eye discs
with Sens and Elav indicate that the ommatidial cluster does not represent the normal
complement of the photoreceptors (compare the arrowheads in Fig. 23D). This suggests that
due to excess recruitment of cells towards the R8 fate the subsequent photoreceptor (R1-R7)
specification is impaired.

As mentioned above, M8 mediates repression of Atonal as a homo/hetero dimer with
other E(spl) repressors. This raises the possibility that M8SA may dimerize with endogenous
M8. Since M8SA does not efficiently interact with Atonal (Fig. 23E), an M8-M8SA dimer
may thus be compromised for repression of Atonal. As a result, an excess number of cells
should adopt the R8 cell fate, as has previously been shown either upon overexpression of
Ato (White and Jarman, 2000) or upon loss of E(spl) (Ligoxygakis et al., 1998). The
supernumerary R8’s associated with M8SA could thus reflect its interference with
endogenous M8, i.e., M8SA might behave as an antimorph. If this indeed the case, the rough
eye of M8SA should be further exacerbated when co-expressed with Ato. This was tested by
the simultaneous overexpression of M8SA and Ato using the driver Gal4^{109-68}. We find that
the rough eye phenotype of M8SA is indeed exacerbated (Fig. 23F). This exacerbation
supports the contention that M8SA interferes with the ability of endogenous M8 to block Ato
(an anitmorphic behavior).
Figure 23. Analysis of the rough eye phenotype of M8SA

Eye imaginal disc from flies expressing M8 (A-B) and M8SA(C-D) were immunoassayed for Senseless (Green) and Elav (Red). Panels B and D indicate the position of R8 (yellow) with respect to the ommatidial cluster (Red). Effect of expression of M8SA in absence (E) or presence (F) of Ato on the eye phenotype as analyzed by light micrographs.
4.4. Autoinhibition by the C-terminal domain and phosphorylation by CK2

The analysis so far indicates that phosphorylation by CK2 mediates conversion of M8 into an active repressor of Ato. CK2 phosphorylates the CTD of M8, thus raising the possibility that in an unphosphorylated state, the CTD of M8 blocks the Ato interaction domain (Orange) maintaining M8 in a ‘protorepressor’ state (Fig. 24). Phosphorylation of the CTD (by CK2) relieves this block and converts M8 into an active repressor. Since M8* lacks this autoinhibitory domain, it is predisposed for enhanced interactions with Ato. The CTD could mediate this autoinhibition by directly occluding the Ato-binding site or by recruiting hitherto unknown cofactor(s). The first of these hypotheses has been tested in vivo by expressing the CTD from wild type M8 (CTD\textsubscript{M8}) or the CTD’s from the CK2 phosphovariants, M8SA (CTD\textsubscript{SA}) and M8SD (CTD\textsubscript{SD}), using the Gal4-UAS approach (Fig. 25).

4.5. Biochemical characterization of the CTD variants

The CTD harbors the invariant C-terminal tetrapeptide, WRPW, which mediates recruitment of the co-repressor Gro (Paroush et al., 1994; Wainright and Ish-Horowicz, 1992), as well as the conserved CK2 phosphorylation consensus. We, therefore, tested to determine whether the isolated CTD’s recapitulate interactions with Gro and CK2 in a manner that is similar to their full-length counterparts. Previous analysis with full-length proteins indicated that the robust interaction of M8 with CK2\textalpha decreased by ~50% with M8SA while M8SD did not interact. In contrast interaction of full-length variants was equivalent when tested against Gro. As expected, all of the CTD variants displayed a robust interaction with Gro (Fig. 26A). In line with the presence of the CK2 consensus, CK2\textalpha interacts robustly with CTD\textsubscript{M8} but not with CTD\textsubscript{SD} (Fig. 26B). In contrast, the interaction between CTD\textsubscript{SA} and CK2\textalpha was comparable to that of CTD\textsubscript{M8} (Fig. 26B). The reason(s) for this discrepancy remains unknown. Nevertheless, the ability of the CTD variants to be
Figure 24. Model for the role of phosphorylation by CK2 in M8 - Atonal interaction.

M8 in unphosphorylated state
Auto-inhibitory C-terminal
domain block Atonal interaction

CK2

Phosphorylated M8

Atonal interacts with
phosphorylated M8

E(spl)D encodes for M8* that lacks
the C-terminus and the potential
'Autoinhibitory' domain.

M8SD mimics phosphorylation
by CK2 thus relieving the orange
domain from the potential
'Autoinhibition' by the C-terminus.

N^spl/Y; E(spl)^D/+  sca-Gal4/+; UAS-m8SD/+
Figure 25. M8 and the CTD variants.

Schematic representation of M8 and the CTD variants.
**Figure 26.** Characterization of CTD variants

A. Interaction of CTD variants

**CK2α**

B. Phosphorylation of CTD variants

A. Interaction of the CTD variants with Gro and CK2α using the LexA based yeast two hybrid system.

B. The indicated GST-fusion proteins were purified, and subjected to phosphorylation using the α2β2 holoenzyme from *Drosophila* embryos. Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie Blue (Gel) and autoradiographed (Film).
properly phosphorylated by CK2 was essentially identical when compared to the full-length protein. For example, only CTD$^{M8}$ was phosphorylated by CK2 in vitro (Fig 26C), suggesting that the isolated CTD’s are not targeted by CK2 in a non-canonical manner.

4.6. Analysis of the autoinhibitory effects of CTD

The autoinhibition hypothesis was tested in vivo via ectopic expression of UAS-transgenes encoding CTD$^{M8}$, CTD$^{SA}$ and CTD$^{SD}$. If the CTD blocks the Orange domain, then expression of CTD$^{M8}$ should interfere with proper functions of endogenous M8 and result in aberrations during eye development. The expression of the CTD$^{M8}$ was carried out either under the scaGal4 driver, or the eyGal4 driver (Annette Parks, Exelixis Inc.)

Expression of CTD$^{M8}$ with scaGal4 did not result in any alteration of the eye field or patterning of the Inter Ommatidial Bristles (IOB’s) (Data not shown). This observation suggests that CTD$^{M8}$ does not interfere with the functions of endogenous M8 during eye morphogenesis. Because scaGal4 mediates expression in the MF (Hinz et al., 1994), at a time point during which R8 specification is underway, it may result in a hysteretic delay in the expression of the CTD. We, therefore, decided to expand the domain of expression anterior to the MF, using the eyGal4 driver. Scanning electron microscopy (SEM) reveals that expression of CTD$^{M8}$ with eyGal4 does not alter the patterning or the size of the eye, but elicits a sporadic duplication of the IOB’s (Fig. 27A). Such a defect is not representative of developmental ‘noise’ because duplicated IOB’s are extremely rare in the wild type. Thus the observed IOB defects could potentially represent interference in M8 function(s).

Based on protein-protein interactions, we have previously found that only M8SD displayed interaction with Ato. This observation led us to propose a model wherein phosphorylation (by CK2) elicited gross displacement of the CTD. This displacement engenders an interaction that is not observed with either M8 or M8SA. If this is the case, ectopic CTD$^{SA}$ should interfere with endogenous M8 during eye morphogenesis, while CTD$^{SD}$ should not. The expression of these CTD’s was carried out either under the scaGal4 driver, or the eyGal4 driver. We find that ectopic CTD$^{SA}$ also elicited a sporadic IOB duplication (Fig. 27B). However, similar effects were also seen with CTD$^{SD}$ (Fig. 27C).

How can such a result be reconciled with our extant model? We believe that the model is oversimplified. We suggest a modification to our model based on conformational
switches that engender/preclude protein-protein interaction(s) while accounting for the observed outcomes of the studies employing ectopic CTD’s. For example, the conformational change in Ras that enable or preclude its interaction with the downstream effector Raf involves a subtle (~2Å) movement of the switch helix. If a similar subtle displacement were to also occur during the switching of M8 from its ‘proto’ to an active repressor state, one would expect all three CTD’s to exhibit somewhat similar effects. If such subtle subdomain movements are, in fact, in play during the switching of M8, genetics might not be the most appropriate means to uncover structural changes. An alternative approach to achieve this goal will be to determine the three dimensional structure of this protein in its nonphosphorylated and phosphorylated forms.
Figure 27. Expression of CTD variants

Eye phenotype due to expression of CTD variants with the driver eyGal4. The genotypes are as indicated. (A-C at 200X and D-F at 1000X)
5. Conclusions: Role of phosphorylation of M8 by CK2.

Repressor activities of M8 during eye morphogenesis appear to be dependent on phosphorylation by CK2. The expression of the phosphomimetic form M8SD dominantly blocks the proneural protein Atonal and leads to a drastic reduction in the eye field. This reduced eye phenotype of M8SD can be reversed by simultaneous overexpression of Atonal indicating that the Atonal is the primary target of M8SD. In addition a partial reversal of the M8SD reduced eye due to decrease in the levels of CK2 provides a strong genetic evidence for the regulatory role of phosphorylation of M8 by CK2. On the other hand, M8SA behaves as an antimorphic protein and interferes with the functioning of M8, thus leading to the enhanced activity of the proneural protein Atonal. Together, these observations provide strong evidence that E(spl) proteins function as dimers in vivo. Preliminary data suggests that the CTD’s might interfere with the function of endogenous M8, but the subtle phenotypes preclude a definitive prediction on the mechanism. Follow up studies in backgrounds mutant for the E(spl) locus or enhanced expression of the CTD’s would be required to determine if the IOB duplications we have observed are indeed relevant.
Chapter 5.

*Drosophila* CK2 phosphorylates Deadpan, a member of the HES family of basic-helix-loop-helix (bHLH) repressors.
1. Abstract

In *Drosophila*, protein kinase CK2 regulates a diverse array of developmental processes. One of these is cell-fate specification (neurogenesis) wherein CK2 regulates basic-helix-loop-helix (bHLH) repressors encoded by the *Enhancer of Split Complex* (*E(spl)C*). Specifically, CK2 phosphorylates and activates repressor functions of *E(spl)M8* during eye development. In this study we describe the interaction of CK2 with an *E(spl)*-related bHLH repressor, Deadpan (Dpn). Unlike *E(spl)*-repressors which are expressed in cells destined for a non-neural cell fate, Dpn is expressed in the neuronal cells and is thought to control the activity of proneural genes. Dpn also regulates sex-determination by repressing *sxl*, the primary gene involved in sex differentiation. We demonstrate that Dpn is weakly phosphorylated by monomeric CK2α, whereas it is robustly phosphorylated by the embryo-holoenzyme, suggesting a positive role for CK2β. The weak phosphorylation by CK2α is markedly stimulated by the activator polylysine to levels comparable to those with the holoenzyme. In addition, pulls down assays indicate a direct interaction between Dpn and CK2. This is the first demonstration that Dpn is a partner and target of CK2, and raises the possibility that its repressor functions might also be regulated by phosphorylation.
2. Introduction

Global signaling pathways are employed in a recurring fashion throughout development to regulate cell-fate specification and differentiation of diverse cell types. The roles of the Notch pathway during neurogenesis, myogenesis, egg chamber formation, etc., is just one notable example (reviewed in Artavanis-Tsakonas et al., 1995; Blaumuller and Artavanis-Tsakonas, 1997; Lai, 2004; Mumm and Kopan, 2000). In *Drosophila*, during the process of neurogenesis, which occurs in the neuroectoderm, Notch orchestrates expression of bHLH transcription factors that either promote (proneural) or restrict (neurogenic) neuronal cell fate (Culi and Modolell, 1998; Dambly-Chaudiere and Vervoort, 1998; Jennings et al., 1994). The former group includes bHLH activators encoded by the *achaete-scute* complex (*ASC*) or *atonal* (*ato*), whereas the latter includes bHLH repressors encoded by the *Enhancer of Split* complex (*E(spl)*C). In the developing eye (for reviews see, Freeman, 1997; Jarman, 2000; Kumar and Moses, 1997; Pichaud et al., 2001; Voas and Rebay, 2004), Notch initially drives expression of Atonal (Jarman et al., 1994; Ligoxygakis et al., 1998; White and Jarman, 2000), and this expression sets up neural competency in groups of cells called proneural clusters. However, with the exception of one cell from this cluster which goes on to adopt a neural fate, others are redirected to an alternate cell fate by expression of *E(spl)*-repressors (Jennings et al., 1994; Ligoxygakis et al., 1998). This inhibitory function of Notch has been termed 'lateral inhibition', and is critical for singling out cells of each proneural cluster that go on to differentiate as neurons (Nagel et al., 1999; Nagel and Preiss, 1999). Thus, interference with the inhibitory phase of Notch elicits supernumerary neurons. In the eye, *E(spl)*M8 antagonizes the transcriptional functions of Atonal via direct protein-protein interactions upon phosphorylation of *E(spl)*M8 (by CK2) at a highly conserved site (Karandikar et al., 2004). As in the eye, bristle morphogenesis also depends on proneural factors encoded by ASC (Achaete, Scute, Lethal of Scute), and their transcriptional functions are similarly antagonized by *E(spl)* repressors (Campos-Ortega, 1998; Heitzler et al., 1996; Modolell and Campuzano, 1998). However, during bristle morphogenesis, phosphorylation of *E(spl)* proteins by CK2 appears to be dispensable (Karandikar et al., 2004). These results raise the possibility that the role of CK2 in regulation of *E(spl)* functions might be context specific.
The E(spl) proteins along with Hairy and Dpn constitute a group of evolutionarily conserved proteins that are collectively referred to as the HES (Hairy and Enhancer of Split) family (Alifragis et al., 1997; Delidakis and Artavanis-Tsakonas, 1991; Maier et al., 1993), and they share some common modes of action. Accordingly, these proteins exhibit a number of conserved domains: a basic domain for DNA-binding, a helix-loop-helix domain for dimerization, an Orange-domain that determines specificity of interactions with proneural proteins, and an invariant C-terminal tetrapeptide, WRPW that recruits the co-repressor Groucho (Fisher and Caudy, 1998). Given the structural similarity of these proteins, we reasoned that CK2 might also regulate Dpn via phosphorylation. In this report, we demonstrate an interaction of CK2 with Dpn.

Dpn is a pan-neural bHLH protein with structural similarities to E(spl)-repressors (Bier et al., 1992; Delidakis and Artavanis-Tsakonas, 1991). However, in contrast to E(spl)-repressors (which are expressed in cells destined for a non-neural fate), Dpn is expressed in neuronal precursors as soon as they are formed and plays important roles during neurogenesis. Consistent with this, loss of dpn has been reported to affect the function but not the gross morphology of the nervous system. Consequently, an absence of Dpn elicits weak motor activity and is lethal (Bier et al., 1992). In addition, Dpn also plays an important role in sex determination (Cline, 1993; Erickson and Cline, 1998). Sex determination, based on the ratio of X-chromosomes to the set of autosomes, initiates in the embryo and involves the functions of three types of genes; X-linked numerator elements such as sis-a, sis-b (scute), and runt, autosomal linked denominator elements (dpn), and maternal factors such as daughterless (da) and extramacrochaetae (emc) (Cline, 1993; Parkhurst and Meneely, 1994). The X/A ratio regulates the activity of a binary switch gene, sex lethal (sxl); when this ratio is 1:1 (females) sxl is active and directs feminization, whereas when it is 1:2 (males) sxl remains inactive. In this context, Dpn acts as a denominator element and one of its functions is to antagonize numerator derived elements, i.e., products of sis-a and sis-b (Scute) via protein-protein interactions (Liu and Belote, 1995). As mentioned above, Scute also functions during neurogenesis. Thus, neurogenesis and sex determination cell-fate determination share some common positive and negative elements for achieving cell fate specification. This supports the prescient suggestion that animal development employs conserved 'functional gene cassettes' reiteratively (Jan and Jan, 1993).
In this report, we demonstrate that another member of the HES family, Dpn, is also a physical partner and target of protein kinase CK2. Dpn contains two sites for phosphorylation by CK2. One of these is positionally conserved in a subdomain of some members of the HES family that regulates their interaction with proneural factors in a CK2-dependent manner. Taking into account the observation that CK2 profoundly influences repressor activity of E(spl)M8 (Karandikar et al., 2004), it would appear reasonable to suggest that this protein kinase might also regulate Dpn functions in vivo. The implications of these findings are discussed.
3. Materials and Methods

3.1. Yeast two-hybrid assay

All manipulations involved in construction of the two-hybrid plasmids were carried out using standard methods, and employed vectors for expression of proteins as C-terminal fusions to the DNA-binding domain of LexA or the activation domain of VP16 (Gyuris et al., 1993). The construction of plasmids expressing Dmα/β as LexA- or AD-fusions has been previously described (Trott et al., 2001b). LexA-Deadpan was a generous gift from Zeev Paroush (Hebrew University, Jerusalem) and has been previously described (Paroush et al., 1994). Two-hybrid interactions were studied in yeast EGY048 containing plasmid pSH18-34. Various combinations of LexA- and AD-fusion plasmids were transformed into EGY048 using lithium acetate, and cultures were analyzed in triplicate for reporter gene (LacZ) expression using a solution-based assay as described (Trott et al., 2001b).

3.2. Purification of GST-Dpn and phosphorylation by CK2

A full length Dpn cDNA was subcloned into the vector pZEX wherein the cDNA is expressed as a C-terminal fusion with GST, and transformed in E.coli BL21. GST and GST-Dpn were expressed and purified essentially as described (Trott et al., 2001b). Following purification, the fusion protein was exchanged into storage buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA, 10% glycerol, 200 mM NaCl, 1mM PMSF) and concentrated using a Biomax-10K centrifugal filter device (Millipore). The concentration and purity were estimated by densitometry of Coomassie stained bands following SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and calibrated using known protein standards.

2 μg of purified GST or GST-Dpn protein were subjected to phosphorylation in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM MgCl2, 10 μM ATP, 5 μCi [γ-32P]ATP and CK2 (1 μg/ml) in a total volume of 40 μl. The reactions were terminated by boiling for 5 min following the addition of 10 μl of 5x sample buffer (Laemmli, 1970). Samples were separated by electrophoresis in 12% acrylamide gels containing sodium dodecyl sulfate, stained with Coomassie, and the destained gels were exposed to Kodak XAR-5 film at room temperature. In order to study the effects of polybasic activators, reactions were supplemented with 0.68 mg/ml spermine, 100 μg/ml poly(DL)lysine, or 125 μg/ml protamine.
3.3. Direct protein-protein interactions

Interactions between Dpn and CK2 were assessed by pull down assays employing GST-fusion proteins and either CK2-holoenzyme or monomeric CK2α. CK2 holoenzyme was purified from embryos according to Glover et al., (Glover et al., 1983), while CK2α was over-expressed via functional complementation of the lethality of \( cka1 \) cka2 \( S. \) cerevisiae, and purified to homogeneity as described (Bidwai et al., 1992). The \( V_{\text{max}} \) of CK2α is 0.4 \( \mu \text{mol/min/mg} \) and that of the \( \alpha_2\beta_2 \) holoenzyme is 1.6 \( \mu \text{mol/min/mg} \) using partially hydrolyzed and dephosphorylated casein as a substrate. These values are similar to those reported earlier (Bidwai et al., 1992; Glover et al., 1983).

Two \( \mu \text{g} \) of purified GST or GST-Dpn were mixed with 25 \( \mu \text{l} \) of glutathione-Sepharose 4B and incubated for two hours at 4 °C. The Sepharose was separated by centrifugation for 1 min at 2000 X \( g \), and the beads were washed twice with 500 \( \mu \text{l} \) of wash buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 5% glycerol, 1 mM PMSF, and 0.1% Triton X-100) to remove unbound GST fusion proteins. The washed Sepharose, containing the immobilized GST fusion proteins, was then incubated with 100 ng of purified Drosophila embryo CK2 and incubated for three hours at 4 °C. The Sepharose was separated by centrifugation for 1 min at 2000 X \( g \), and the supernatant was recovered as unbound material. The pellets were washed two times for 5 minute each, with 500 \( \mu \text{l} \) of wash buffer. Sepharose-bound (pellet) and unbound (supernatant) fractions were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. CK2 was detected by Western blot analysis using primary antibody against CK2 at a dilution of 1:1000 and secondary antibody (goat-anti-rabbit IgG coupled to alkaline phosphatase, Bio-Rad) at a dilution of 1:3000. Immunoblots were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indooyl phosphate.
4. Results and Discussion

4.1 A positionally conserved CK2 site in HES-repressors.

We had previously observed, using the yeast two hybrid assay, that a subset of E(spl)-repressors, i.e., M5, M7, and M8, robustly interact with CK2α (Trott et al., 2001b). In addition, these three proteins are equivalently phosphorylated by monomeric CK2α or the holoenzyme at a conserved CK2 site that is located in close proximity to the C-terminal Groucho binding WRPW motif (Fig. 28A). Furthermore, deletion of the CK2 site (SDCD) or replacement of the CK2 phosphoacceptor in M8 with Asp abolished interaction, suggesting that the CK2-site might, by itself, confer interaction. Given the overall structural conservation of the HES family, i.e., E(spl), Dpn, and Hairy, we, therefore, analyzed the sequence of Dpn to determine the presence of CK2 sites and their positional conservation, if any. This analysis revealed the presence of two potential sites, i.e., S9DDD and S408DCS411LDE (Fig. 28A). While the N-terminal site satisfies the requirement for an Asp/Glu at the n+1 and n+3 positions, the C-terminal site is lacking Asp/Glu at the n+1. However, we note that a number of substrates where the n+1 position is not an Asp/Glu have been identified (reviewed in Meggio and Pinna, 2003). In Dpn, the first site is adjacent to the basic domain and harbors a single potential phosphoacceptor (Ser9). In contrast, the second site is located in the vicinity of the Groucho binding WRPW motif and contains two potential phosphoacceptors (Ser408 and Ser411) that might be subject to hierarchical phosphorylation by CK2. Interestingly, the second site localizes to a region of Dpn which, although hypervariable amongst HES members, is positionally conserved in a number of repressors (M5/7/8, Hes6, etc.) (Karandikar et al., 2004). In case of M8 and its murine homolog Hes6, this site is targeted by CK2 in vitro, and its perturbation dramatically affects their repressor activity in vivo (Gratton et al., 2003; Karandikar et al., 2004; Trott et al., 2001b).

4.2 Two-hybrid interaction of CK2 and Dpn.

Given the strong two hybrid interaction of E(spl)M5/7/8 with CK2, and that interaction required integrity of the CK2 site, we sought to ask whether Dpn was also a partner of CK2. However, in an explicit test we observed that strength of the (two hybrid...
Figure 28. Functional motifs in HES repressors.

**A.** Schematic representation of the functional motifs common to HES bHLH proteins. The structural/functional motifs are: basic domain (black box with halo), HLH and Orange domains (gray boxes), the C-terminal tetrapeptide, WRPW, that binds Groucho (black box), and the CK2 consensus site (checkerboard box). Size heterogeneities between HES members is not indicated. Inset shows the alignment of the sequences encompassing the CK2 site of a subset of HES members, and asterisk denotes the CK2-phosphoacceptor.

**B.** Comparative two hybrid interactions of E(spl)m5/7/8 and Dpn with CK2α. Yeast EGY048 harboring the LacZ-expression plasmid, pSH18-34, was transformed with plasmids expressing the indicated protein pairs. Transformants were grown in galactose medium, and the levels of LacZ were determined as described (Trott et al., 2001a). LacZ activity is expressed in Miller Units, and the data shown is the average of 3 independent experiments.
interaction between LexA-Dpn and AD-CK2α appeared marginal when compared to that between LexA-M8 and AD-CK2α (Fig. 28B). This result was surprising because Dpn contains two CK2 sites, both of which are significantly more acidic than the single site in M5/7/8 (see Fig. 28A). We reasoned that the significantly attenuated Dpn-CK2α interaction might reflect attenuated expression and/or instability of Dpn in yeast, or, perhaps, its ability to act as a repressor in yeast. An alternative possibility is that this interaction also requires CK2β (see below). If so, a direct biochemical route might be more informative to assess targeting of Dpn by CK2.

4.3 Deadpan is phosphorylated by CK2.

To test if Dpn is a CK2 target we performed an in vitro phosphorylation assay. GST and GST-Deadpan were subjected to phosphorylation using purified monomeric CK2α or CK2 holoenzyme. The former isoform is relevant to our two hybrid analysis, whereas the latter isoform mimics the environment most likely to be encountered in vivo and thus might be considered to be physiologically more relevant. Our results indicate that GST-Dpn is phosphorylated weakly by monomeric CK2α, whereas it was robustly phosphorylated by the embryo-holoenzyme (Fig. 29). No phosphorylation of the GST affinity tag was observed for either isoform of CK2. These results suggest that phosphorylation of Dpn by CK2 is positively influenced by the β subunit, and might explain its ‘weak’ interaction with CK2α in yeast. We do not consider it likely that Dpn interacts exclusively via CK2β, because CK2α exhibits phosphorylation of this bHLH protein, albeit weakly. The more likely scenario is that the Dpn interacts with CK2 via a binding site encompassing both subunits, i.e., the holoenzyme. Because this is the in vivo conformation of CK2 strengthens the notion that Dpn is a CK2 target. Comparative kinetic analysis with the two isoforms will be needed to address how CK2β enhances interaction and phosphorylation of Dpn.

4.4 Direct interaction of Dpn and CK2.

Although the phosphorylation analysis suggest that Dpn interacts preferentially with the holoenzyme, two hybrid analysis with this isoform per se has been precluded because yeast strains that express equivalent amounts of CK2α and CK2β are currently unavailable. We have, therefore, assessed the ability of Dpn to form a direct complex with embryo-CK2
The indicated GST-fusion proteins were purified, and subjected to phosphorylation using the monomeric $\alpha$ subunit, CK2$\alpha$ or the $\alpha_2\beta_2$ holoenzyme from Drosophila embryos. Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie Blue (Gel) and autoradiographed (Film). The positions of GST and GST-Dpn are denoted by arrows, and the arrowhead indicates the autophosphorylated CK2$\beta$ subunit.
Figure 30. Interaction of Dpn with CK2α or CK2-holoenzyme.

Bacterially expressed GST alone or a GST-Dpn fusion protein were immobilized on glutathione-Sepharose beads, and incubated with either monomeric α subunit (CK2α), or the α₂β₂ holoenzyme from *Drosophila* embryos (CK2Holo). The beads were separated from the unbound material, and the bead bound (P, pellet) and the unbound (S, supernatant) samples were examined for the presence of CK2 by Western blotting. The arrows indicate immunoreactive bands corresponding to CK2α and CK2β.
or CK2α. GST-alone and GST-Dpn were purified, immobilized on glutathione-Sepharose, and tested for complex formation with the two isoforms of CK2. The presence of CK2 in the bound (pellet) and unbound (supernatant) fractions was assessed by Western blotting using an antisera which recognizes both (α and β) subunits of CK2 (Dahmus et al., 1984). As expected, incubation of CK2 with Sepharose beads did not result in immunoreactive material in the pellet fraction (data not shown). In addition, incubation of GST-beads (Fig. 30, lanes 1 and 3) also did not result in any immunoreactive material in the pellet, indicating that neither isoform interacts with GST consistent with their inability to phosphorylate this affinity tag (see Fig. 29). Incubation of GST-Dpn beads with CK2α resulted in a minor amount of immunoreactive material in the pellet (Fig. 30, lane 5). In contrast, incubation of GST-Dpn beads with embryo-CK2 (Fig. 30, lane 7) resulted in significantly greater amounts of immunoreactive material in the pellet, demonstrating that Dpn and CK2-holoenzyme interact directly. These binding data appear to qualitatively mirror the phosphorylation data (see Fig. 29), and we estimate that ~20% of the holoenzyme interacted with Dpn. Given the experimental conditions of these assays, CK2-holoenzyme contributed half the amount of catalytic subunit compared to CK2α alone, suggesting that complex formation appears to be relatively efficient for the holoenzyme. These results demonstrate that the Dpn-CK2 interaction is direct. In addition, complex formation occurs in the absence of Mg-ATP, in line with previous analysis of the interaction of this enzyme with m5/7/8, ZFP35, etc. (Kalive et al., 2001; Trott et al., 2001a).

### 4.5 Effect of polybasic compounds on the phosphorylation of Dpn.

Our observations of a direct CK2-Dpn complex and its preferential phosphorylation by the holoenzyme, suggested for a positive role for CK2β. The marginal ability of CK2α to phosphorylate Dpn (see above), and that CK2β mediates activation by polybasic effectors (Bidwai et al., 1993; Meggio et al., 1994a), led us to assess whether phosphorylation was responsive to polybasic activation. The marginal phosphorylation of Dpn by CK2α was unaffected by either spermine or protamine, but was dramatically stimulated by poly(DL)lysine (Fig. 31, compare lanes 5-8). The stimulatory effects of poly(DL)lysine are not due to non-specific phosphorylation, because GST is not phosphorylated in its presence (Fig. 31, compare lanes 1 and 3). In contrast, phosphorylation of Dpn by embryo-CK2 was
Figure 31. Effect of polybasic activators on phosphorylation of Dpn.

GST-alone or GST-Dpn were purified, and subjected to phosphorylation using the monomeric α subunit (CK2α) or the α2β2 holoenzyme from Drosophila embryos. Samples were phosphorylated with either CK2α (lanes 1-8) or the holoenzyme (lanes 9-16). Phosphorylations were conducted in the absence of any effector (lanes 1, 5, 9, and 13), or in the presence of 0.68 μg/ml spermine (lanes 2, 6, 10, and 14), 100 μg/ml poly(DL)lysine (lanes 3, 7, 11, and 15), and 125 μg/ml protamine (lanes 4, 8, 12, and 16). Samples were electrophoresed in 12% SDS-polyacrylamide gels, stained with Coomassie Blue (Gel, upper panels), and autoradiographed (Film, lower panels).
unresponsive to further activation by these effectors (Fig. 31, lanes 13-16). These results suggest that phosphorylation of Dpn by embryo-CK2 is unresponsive to further activation, and supports the notion that substrates that are efficiently phosphorylated, e.g., the RII subunit of PKA, Topoisomerase II, etc., are generally refractory to these activators (Bidwai et al., 1993).

**4.6 Implications of Phosphorylation of Deadpan.**

While the mechanism by which Dpn functions during neurogenesis remains to be resolved, its role(s) during sex determination are much better understood. In either case, however, one common feature of its functions is antagonism of ASC, whereby Dpn represses transcription of ASC via DNA-binding (Winston et al., 1999). In line with this, ectopic expression of dpn reduces ASC activity, suggesting a negative interaction between these two loci. It is noteworthy that a similar function is ascribed to HES repressors as well, although in their case DNA-binding as well as direct interactions with proneural factors (ASC and Atonal) are known to be required for antagonism (Alifragis et al., 1997; Giagtzoglou et al., 2003; Nagel and Preiss, 1999).

How might phosphorylation of Dpn regulate its in vivo functions? It is difficult to propose this with certainty based solely on in vitro analysis. However, based on the extensive body of genetic and molecular analysis on Dpn to date, and the emerging notion that CK2 profoundly influences the activity of the related repressor, E(spl)M8, during eye development (Karandikar et al., 2004), some possibilities can be predicted. As stated above, CK2 phosphorylation regulates repressor activity of M8 and replacement of the phosphoacceptor with Asp generates a dominant allele that is severely exacerbated for its antineurogenic functions. A similar CK2 dependent mechanism might also underlie the interaction of M8 with the ASC-bHLH activator, Lethal of Scute (Karandikar and Bidwai, unpublished). In a similar vein, it is conceivable that phosphorylation of Dpn might augment its ability to antagonize ASC-derived bHLH activators by either modulating DNA binding or direct protein-protein interactions. CK2 is known to regulate DNA-binding as well as protein-protein interactions (Gratton et al., 2003; Karandikar et al., 2004; Luscher et al., 1990; Luscher et al., 1989).
4.7 bHLH repressors and CK2, a recurring theme during neurogenesis.

The development of nervous system is regulated by the interplay between proneural proteins and their repressors. A general strategy during neurogenesis appears to be the conferring of neural potential on a field of cells, from which arises a precise pattern of neural and accessory cell fates through this interplay and, as such, this mechanism also appears to be involved in other cell fate decisions. It is increasingly becoming apparent that cell fate choice is unlikely to be based simply on the levels of an activator versus its cognate repressor. Rather, this interplay must also be modulated in a spatial and temporal context. In such a scenario, regulation of protein turnover, presence or absence of cofactors, and regulatory modifications, etc., might provide a means to achieve 'fine tuning' of this interplay. In this context, protein kinases and/or phosphatases might provide a simple bistable mechanism to 'fine tune' the developmental outcome (Modolell and Campuzano, 1998). Such a mechanism is beginning to emerge for regulation of repression by E(spl)M8 and its mammalian counterpart, Hes6 (Gratton et al., 2003; Karandikar et al., 2004). In both, phosphorylation by CK2 regulates their ability to interact with and antagonize proneural factors. Given the expanding repertoire of HES proteins that are targeted by CK2, it would not come as a surprise that a similar mechanism might also be employed for regulation of another HES member, Dpn.

Among the HES members that are CK2 targets, Dpn differs from E(spl) in a number of ways. While E(spl) transcription (via Su(H)) occurs in response to an activated Notch receptor, Dpn has been thought to be Notch-independent, although it contains binding sites for Su(H) in a region that recapitulates PNS/CNS specific expression (Emery and Bier, 1995; Rebeiz et al., 2002). Furthermore, E(spl) repressors block proneural proteins in cells undergoing lateral inhibition, whereas Dpn achieves a similar outcome but in neural cells (Campos-Ortega, 1998; Deshpande et al., 1995; Modolell and Campuzano, 1998; Wrischnik et al., 2003). The remarkable conservation of CK2 by itself (reviewed in (Bidwai, 2000; Glover, 1998), and its ability to modulate the activity of repressors in different developmental contexts might be indicative of its selection as a general modulator of cell fate determination.
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